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EXPERIMENTS IN THE GRAFTING OF SPECIES IN THE GENUS VIOLA[1]

JOHN D. DODD[2] AND ALEXANDER GERSHOY[3]

INTRODUCTION

Cyto-taxonomical and hybridization studies of the genus *Viola* have been in progress at the Vermont Agricultural Experiment Station for a number of years. The norm of hybridization limits among the species in the various sections and sub-sections of this genus have been determined (Gershoy, 4). No intersectional crosses have been consummated although hybrids between species representing the distinct subsections of the *Nominium* section have been grown to maturity. The present investigation represents an attempt to determine what relationship exists between the capacity for hybridization and the capacity for grafting in this genus.

In order to test for a positive correlation it is necessary to demonstrate that grafting limits lie within the violet genus as represented by the species available for study. Evidence in the literature of graftage suggests that the limits are not generally confined to so small a range as a genus:

A. Mirov (8), in a recent paper, described experiments in which he found no difficulty in grafting various species of pine.

B. Intergeneric grafts in many families, particularly in the *Solanaceae*, have been an object of study by numerous workers, many of them interested in chimaeras. Jones (5) has discussed the work of Winkler, Baur, and others in this respect.

C. Daniel (3), in a review of the literature and in a discussion of his own work, had earlier shown that graft combinations are possible between unlike species of plants separated, in some cases, by greater than generic differences.

A general statement of the problem may perhaps be reduced somewhat to the following query: does a relationship exist between, on the one hand, the ability of two different protoplasms to survive in the close juxtaposition of tissues such as is afforded in a graft union and, on the other hand, the capacity of these unlike protoplasms to achieve a successful fusion of gametes?

In regard to the problem of grafting limits it should be emphasized here

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that the violet genus as recognized by Engler and Prantl (1) covers a very wide range of morphological types. It is not considered improbable, therefore, that intrageneric limits might exist. So far as the authors are aware a study of experimental graftage has not been made in the violet genus.

EXPERIMENTAL PROCEDURE

Practical considerations limited the experimental program to the determination of a fair sample of the reciprocal grafting combinations that are possible with available species. Five species were chosen for use as being representative of the wide morphological, anatomical, and physiological differences existing among the north temperature members of the genus.

DESCRIPTION OF SPECIES TYPES USED[4]

Section *Melanium:* *V. tricolor* var. *florairensis* (?) or var. *lutea* of horticultural origin (?).

This species is potentially biennial but often behaves as an annual. The axes of first and second order are erect and flowering. Flowering is continuous throughout the season. Except in the basal nodes of older stems the proportion of parenchymatous tissue is high and mechanical tissue seems to be limited to the xylem elements in the separate vascular bundles. The pith cells are ruptured by stem enlargement, resulting in hollow stems.

Section *Chamaemelanium: V. canadensis* L.

This is a perennial species. The primary axes form short, thick, fleshy rootstocks which are transversely geotropic. Secondary axes are erect and flowering. Each aerial shoot is herbaceous and exhibits rapid growth of comparatively short duration. Leaf- and flower-size diminish markedly as meristem activity dwindles. Mechanical tissue is limited to the xylem elements in the separate bundles and the proportion of parenchymatous tissue is high. Usually the pith cells are not enlarged beyond elasticity limits as judged by a lack of schizogenous areas. They are capable of some regenerative activity.

Section *Nominium,* subsection *Rostellatae: V. striata* Ait.

The basal portions of axes of first and second order become erect or inclined woody rootstocks of at least biennial duration. Flowering axes are erect or inclined. They are herbaceous and have an intact pith. There is a layer of presumably pericyclic fibers which serves to isolate the phloem tissue of each bundle from the parenchyma cells of both the cortex and the medullary rays. New leaves and flowers are developed throughout the season.

Section *Nominium,* subsection *Uncinatae: V. odorata* L.

Primary axes are quite short, erect and at least of biennial duration. In

[4] The taxonomical descriptions are adapted from Gershoy (4). Certain anatomical and histological features are added by the authors in the present paper.

such axes the amounts of cortical and pith parenchyma are relatively small. Vascular tissue becomes a complete cylinder resulting in a high proportion of mechanical conducting cells. Lateral axes form short or long, leafy runners, potentially capable of rooting at any node. The anatomy of these runners is similar to that of the primary axes although there is not as much mechanical tissue present. A terminal rosette develops into a short, erect axis resembling the primary seedling axis.

Section *Nominium*, subsection *Plagiostigma: V. papilionacea* Pursh.

Primary axes are foreshortened into thick and fleshy rhizomes which form similar, secondary axes in the axils of the leaves. Continual branching and separation of branches results in clonal clusters. Leaves are long-petioled and have broad, cordate-ovate blades. Parenchymatous tissue is inclined to be mucilaginous in older plants, particularly in the rhizome. Flowers develop in the leaf axils of the seedling rhizomes first.

The program of effecting reciprocal grafts among these five species was carried out during the summer growing seasons of 1939 and 1940. Since the north temperate species of violets grow very slowly and abnormally under warm greenhouse conditions in winter they are left in a dormant state. For this reason the study was limited to the months from May to September, i.e., between the times when the leafy branches were large enough to be used and when they became decadent in the fall.

During these two summers several hundred grafting operations were performed in an attempt to develop technics which would permit successful grafting of species with such varied morphology as those selected for study. Many of these experimental grafts were unsuccessful. However, as will be indicated elsewhere in this paper, individually successful grafts encompassed most of the planned intersectional combinations.

It was tentatively planned to attempt each interspecific combination a definite number of times and then to compare the numbers of the successes of each combination, thus putting the results on a statistical basis which might permit a conclusion as to the degree of success of any given interspecific combination.

However, it is obvious from the following considerations that such data as were obtained do not lend themselves to worthwhile statistical analysis:

1. Since a successful graft indicates that a given type combination is possible all of the unsuccessful attempts to obtain a like combination may be the result of faulty technic.

2. The successful technics were developed by the trial and error method and varied markedly from each other. It has not been possible to enlarge the experimental program in order to analyze statistically the variables involved

most closely in grafting success. Some of these variables are mentioned briefly as follows: a, time of grafting; b, relative ages of tissues in contact; c, relative areas of tissues in contact; d, pressure of bindings and protection of the graft region.

EXPERIMENTAL TECHNIC

The actual grafting technics were varied for each combination of species. The reciprocals of each interspecific combination often differed from each other, in methods used as well as tissues placed in juxtaposition. In general, however, the methods used can be classified into two well known types: sleeve grafts and whip grafts. Approach grafts were tried and abandoned since they gave poorer results than simple whip grafts and could only be used for the same types of species combinations. Bud grafts would have involved the manipulation of extremely delicate tissue and were not considered practical under the experimental conditions. Bottle grafting, as described by Blakeslee and Farnham (2), is an interesting method which, unfortunately, escaped the authors' attention until the present experiments were concluded. It will be tested later.

Seedling grafts presented a definite obstacle to many of the desired grafts since such grafts would have required a union between the swollen and fleshy primary axes of such species as *V. papilionacea* and the slender, relatively unstrengthened axes of such species as *V. tricolor*. It is impossible, in such cases, to adequately match the areas of wound surfaces. However, the seedlings of *V. papilionacea* were easily grafted to mature stems of *V. tricolor*. The hollow stems of the latter species were split open lengthwise and laid flat against the cut surface of the fleshy rhizome of the former, thus completely matching the cut surface.

In order to maintain a degree of uniformity in methods it was decided to reserve the method of seedling grafts as a separate problem and to use it in the present investigation only as a last resort when experiments with mature plants failed to yield a desired interspecific graft.

Cuts for the graftage were made with a sharp, clean razor. The stock and scion were tied together with strips of moistened cellophane tape which was found to be easier to manipulate than raffia. Each graft was bound with a narrow strip of Parafilm which was wrapped around the graft region in much the same manner as a tape bandage. The warmth of the fingers sufficed to form an adequate seal. This bandage was removed after about three weeks. A shade frame was used during the first week but it was found that continued close shading induced conditions favorable to mildew, particularly in grafts which involved *V. tricolor*, a species intolerant of shade.

INTERPRETATION OF RESULTS

At the beginning of this project it became obvious that the interpretation

of results would present a major problem. Fruit trees and ornamentals are grafted with a definite end in view which, if attained, indicates success in grafting. The present problem, however, did not lend itself to a single and immediately obvious criterion of grafting success and the selection of arbitrary tests which would define a fully successful graft became a central point in the interpretation of the results obtained.

After careful consideration it was decided that a perfect graft could be defined as one which either possessed the following characteristics or gave indications that these characteristics would be obtained were the graft carried into another growing season:

1. The graft must form a tissue union in which living cells of each member would be in immediate contact, that is, there should be no layer of necrotic tissue left between the stock and scion. Vascular continuity should be established between the stock and scion in such a tissue union.

2. The root system of the stock and the shoot system of the scion should both continue vigorous growth. The scion should develop vegetative parts of normal appearance from primordia which are laid down at some time after the grafting operation. It is necessary to make a careful distinction between continued growth of new primordia and the enlargement of parts in existence previous to grafting.

From a theoretical point of view it should be proven that the stock has utilized food material passed across the contact region by normal conduction mechanisms. The use of seedling stocks would provide a direct demonstration of this since there would be comparatively little reserve food in the primary axis and there would be no secondary axes of stock origin which would serve to feed the root system. Unfortunately, the previously explained limitations to the use of seedling stocks have prevented such a direct physiological criterion.

Some indirect evidence has arisen that stock tissue has utilized scion foodstuffs. An example of this is the graft of *V. odorata* on *V. tricolor* stock. In this graft only one of the vascular bundles in the stock formed vascular continuity with the scion. It enlarged, through cambial action, to such an extent that it became comparable, in size, to the stele of an entire normal stem. Since all connection with the original stock shoot system had been severed it seems a plausible explanation that scion foodstuffs were utilized in this hyperplastic growth of the stock.

3. Daniel's (3) postulate that, in a successful graft, the scion should mature fruit, seems to be a sound one. In order for fruiting to take place a graft union must form and the scion must continue growth. It should be emphasized again, here, that buds of the scion in existence at the time of grafting may develop to some extent without the formation of a true union. In no such case, however, have the buds shown the beginnings of swollen ovaries.

The first two criteria of grafting success could be presupposed from data showing that the scion had developed fruit from the primordia arising subsequent to graftage. Fruiting could, then, be used as a single criterion. However, it has not always been possible to adopt this test in the present work because many of the developing grafts were cut short by the advent of adverse growing conditions, coincident with the beginning of the winter period of dormancy for violets. Thus, these grafts did not survive long enough to develop fruit.

Attempts to overwinter some of the grafts, both on the original stock plants and as a form of cutting, met with failure. The apparent success of a few overwintered grafts was shown to be due to the formation of roots by the scion species.

From the preceding discussion it is evident that in the determination of its degree of success each attempted graft combination is to be treated separately. The attempted grafts are discussed individually under one of the following classifications:

A. Successful grafts.
B. Unsuccessful grafts.
C. Special cases.
   I. Grafts involving *V. canadensis* as the scion.
   II. Grafts involving *V. papilionacea* as the stock.

A. SUCCESSFUL GRAFTS

1. *V. papilionacea* on *V. tricolor* stock (fig. 3).

A seedling rhizome of the scion species was sliced diagonally so that the apex remained uninjured. The stock was decapitated and split open lengthwise on one side at the tip of the stump. The inner surface of the hollow stump was pressed against the cut surface of the scion and the graft was bound in the manner previously described.

A graft union formed and the scion developed several leaves to a normal size. No fruit was formed during the duration of this graft but the extreme vigor of growth of the scion warrants its being termed successful. Mature rhizomes of *V. papilionacea* were tried with no success, the failure being perhaps due to the inhibiting action of the mucilaginous substance which exuded from cut surfaces and which prevented a direct cell-to-cell contact between the stock and scion.

2. *V. papilionacea* on *V. canadensis* stock.

A seedling of the species used as the scion was prepared in the same manner as in the preceding graft. The stock was decapitated at the second node and a diagonal slice of such a length that the cut surface matched that

of the scion was made through the region of the node. The injured surfaces were placed together and the graft was bound.

A graft union formed and the scion developed several new leaves. It was considered, also, to be a potentially successful graft although the growth was not quite as luxuriant as in the preceding case.

3. *V. odorata* on *V. tricolor* stock (fig. 6).

The stock was decapitated and split open at the second node. A portion of a runner of *V. odorata* with a developing rosette at the tip was used as the scion. The runner was partially decorticated for about an inch and the cut surface was fitted into the hollow portion of the split internode. The scion formed several new leaves and maintained a normal appearance. No flower buds were developed before the end of the growing season. A complex graft union formed in which a vascular bundle of the stock went through a period of hyperplastic enlargement. This enlargement has been discussed previously as evidence that the stock was able to utilize scion foodstuffs.

4. *V. striata* on *V. canadensis* stock (fig. 1).

The stock was decapitated at the second node and a diagonal slice, two inches long, was made up through the internode below to the node where the shoot was severed. A four-inch tip of a lateral flowering shoot of the scion parent was cut off at the nearest convenient node. A diagonal slice was made from this basal node to the node above. The graft was bound in the usual manner.

The scion continued to develop new leaves of normal appearance throughout the summer and finally matured a small pod with one seed. This is a very small percentage of the normal seed number.[5]

5. *V. odorata* on *V. canadensis* stock (fig. 4).

The stock was decapitated at the second node and sliced diagonally as in no. 4. A portion of a runner of *V. odorata* with a terminal rosette was used as the scion. This runner was sliced diagonally so that its cut surface matched that of the stock.

A graft union formed and the scion continued normal growth. Two seed pods were matured during the rest of the season. There was one seed in each pod.[5]

6. *V. striata* on *V. odorata* stock (fig. 5).

A three-inch tip of the scion plant was cut off at the nearest node and sliced diagonally. The terminal rosette of a runner of *V. odorata* was cut off

[5] Manch (8) recorded the normal seed number for several species of violets. The seed numbers of all pods formed by scions in the present experiments were small percentages of the normal number for the species used as scions.

and the runner was used as the stock in a typical whip graft. A union formed and the scion continued to develop new leaves of normal size throughout the season. No fruit was matured and no flower buds were observed.

Although *V. odorata* has formed several unions as a scion this is, so far, the only successful graft in which this species has served as a stock.

7. *V. odorata* on *V. striata* stock (fig. 2).

A runner with terminal rosette was used as the scion. The stock was treated in the same manner as the stock in no. 5. The graft was a typical whip graft.

A union formed and the scion continued normal growth. Two seed pods were matured towards the end of the season, each containing one seed.

8. *V. tricolor* on *V. canadensis* stock.

The stock was decapitated at the second node and its cortex was removed over a length of two inches and about two thirds of the circumference. A four-inch tip of a flowering shoot of *V. tricolor* was split lengthwise at its base and fitted as a sleeve around the stump of the stock.

A graft union formed and slight vegetative growth occurred. Flower buds in evidence before the graft developed into flowers but did not develop fruit. However, *V. tricolor* does not self-pollinate readily by mechanical means although it is not self-sterile. This graft was regarded as being only moderately successful.

B. UNSUCCESSFUL GRAFTS

The grafts listed below were attempted several times, with varied technics, without success. However, the number of attempts was not statistically large enough to warrant the generalization that grafting is not possible in these cases. Special technics for these combinations have not been devised as yet.

1. *V. tricolor* on *V. odorata* stock.

The reciprocal of this, however, was successful.

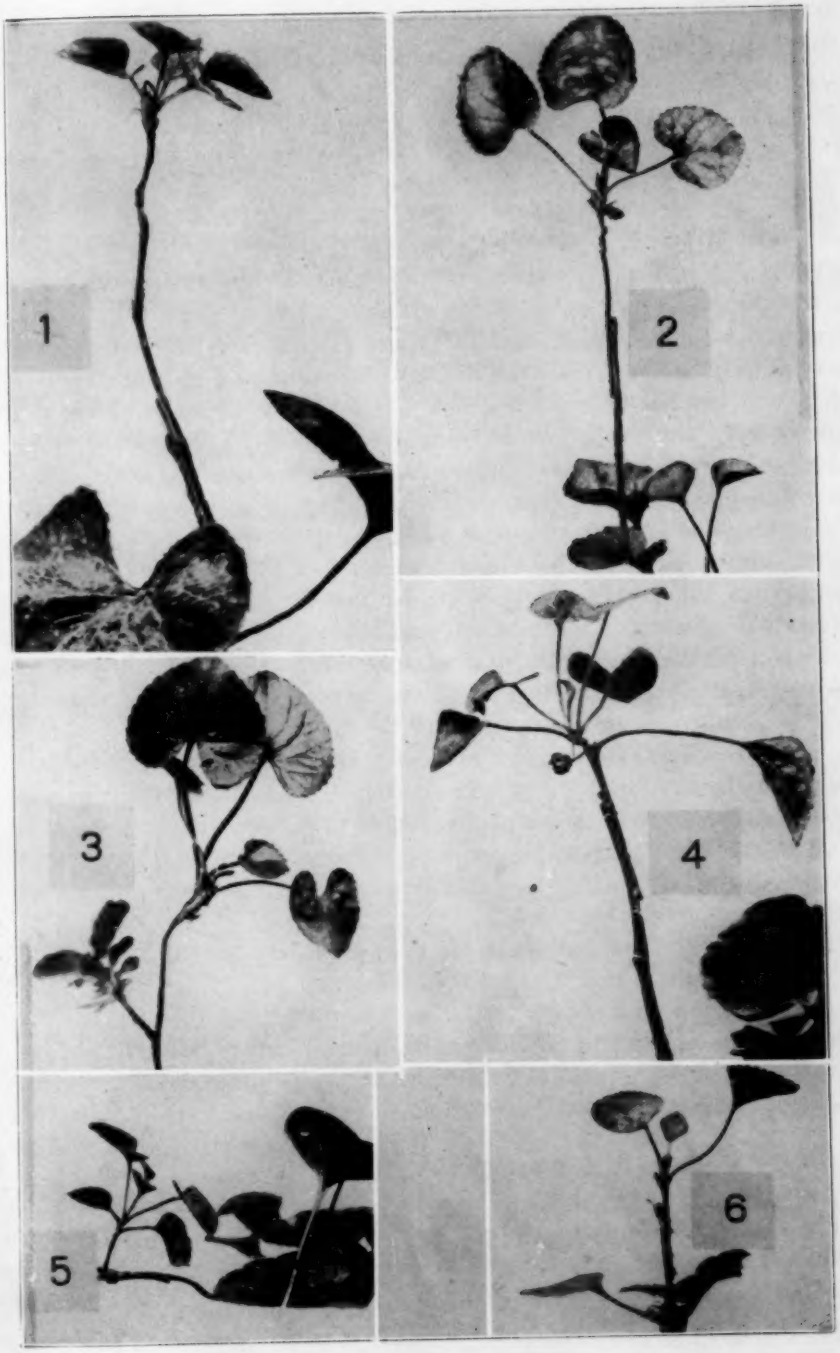
2. *V. papilionacea* on *V. striata* stock.

The reciprocals of grafts (2) and (3) are discussed under Special Cases, part 2.

3. *V. papilionacea* on *V. odorata* stock.
4. *V. tricolor* on *V. striata* stock.
5. *V. striata* on *V. tricolor* stock.

Explanation of figures 1–6

FIG. 1. *V. striata* on *V. canadensis* stock. FIG. 2. *V. striata* on *V. odorata* stock. FIG. 3. *V. papilionacea* on *V. tricolor* stock. FIG. 4. *V. odorata* on *V. striata* stock. FIG. 5. *V. odorata* on *V. canadensis* stock. FIG. 6. *V. odorata* on *V. tricolor* stock.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C. SPECIAL CASES

Several of the grafts attempted could be classed neither as successes nor, definitely, as failures. Certain morphological characteristics of one of the species involved made it difficult to devise technics of grafting which would give satisfactory results from the point of view of the arbitrary criteria previously discussed. These questionable results are here grouped together.

1. Grafts involving *V. canadensis* as the scion.

It has not as yet been possible to induce *V. canadensis* to continue growth as a scion, either as a self graft or with other species as the stocks. Mature branches form homoplastic graft unions readily and do not deteriorate prematurely. But even in such self grafts they do not continue to develop new parts. As has been pointed out previously the aerial branches of this species have a rapid growth-phase of short duration. The growth of these branches as scions in a graft as well as in the undisturbed shoot is highly determinate in the sense that meristem activity dwindles, which causes the size of leaves and flowers to fall off rapidly near the end of growth. Attempts have been made repeatedly to graft the shoots of *V. canadensis* in the growing period. However, they are so tender that they wither quickly in spite of shade protection. It is considered possible that the technic of bottle grafting may contribute pertinent data to this problem since it might permit the use of immature branches by giving them an accessory supply of water until they establish vascular continuity with the stock.

From the above discussion it is evident that only one and, on the whole, an unsatisfactory criterion of success could be used here, namely that of graft union formation by mature branches when used as scions.

The three grafts listed below existed for a period of more than two months during which time no noticeable changes in the scion, other than a slight chlorosis, were recorded.

V. canadensis on *V. tricolor* stock, a sleeve graft.

V. canadensis on *V. striata* stock, a whip graft.

V. canadensis on *V. odorata* stock, a whip graft.

The remaining graft combination, involving *V. canadensis* as the scion, in which attempts were made to graft it on *V. papilionacea* stocks is discussed below under Special Cases, part 2.

2. Grafts involving *V. papilionacea* as the stock.

As explained above this species does not produce erect, aerial shoots or runners of long-continued growth which could be used easily as stocks. Attempts to use mature rhizomes were uniformly unsuccessful. The presence of large amounts of mucilage secreted by injured rhizomes has been suggested as inhibiting a direct cell-to-cell contact between stock and scion.

The leaf petioles of this species are long and sturdy enough to warrant their attempted use as stocks. The amount of mucilage secreted by injured petioles is comparatively small and in several instances graft unions formed when petioles were used as stocks. A few parenchymatous cells were observed to have been transformed into xylem elements but vascular continuity was not established between stock and scion. Moreover, the scions merely existed without change, none of them showing any indications of continued growth. These grafts are interesting, however, from the point of view of the proliferation of cells which occurred. Such a proliferation perhaps indicates that successful grafts are possible when *V. papilionacea* is used as a stock, providing an adequate technic can be devised.

The following species were used as scions in grafts upon leaf petioles of *V. papilionacea*. The general description immediately above applies equally well to all of the resulting grafts: *V. canadensis, V. tricolor, V. striata, V. odorata.*

TABLE I. *Results of Grafting Experiments in the Genus Viola*

Stocks / Scions	*V. striata*	*V. odorata*	*V. papilionacea*	*V. canadensis*	*V. tricolor*
V. striata		+	O	S	O
V. odorata	+		O	S	O
V. papilionacea	S	S		S	S
V. canadensis	+	+	+		+
V. tricolor	O	+	+	S	

+ Indicates a successful graft.
O Indicates a failure not due to observed technical difficulties.
S Indicates a failure which may be due to technical difficulties and is discussed in the text under Special Cases.
Self grafts have not been included in the present study.

CONCLUSIONS

The results briefly summarized in table 1 indicate that seven out of the ten graft combinations possible among the selected representative species in each of the distinct sections and sub-sections of the genus *Viola* have been successfully consummated in at least one of the reciprocal stock and scion relationships. It may be inferred from these results that the failure of one reciprocal can find no assumed basis in phylogenetic divergences if the other reciprocal has been obtained.

It is postulated further that the failures listed above are due to technic rather than to so-called grafting incompatibilities arising in some way from

phylogenetic divergence. A discussion of possible reasons for this technical failure has been presented in the text.

The conclusions presented are (a) that grafting limits are not coincident with any morphological divisions of the genus such as those presented by Engler and Prantl (1); and (b) that there are probably no general grafting limits within the genus *Viola*. It would, of course, be an impracticable task to demonstrate the presence or absence of specific grafting imcompatibilities which might exist between any of the large number of species in the genus, regardless of their relationships. The one example in the present work which is suggestive of such a condition is the failure of *V. striata* and *V. tricolor* to form a graft union in either reciprocal combination. This example, however, should be investigated to a fuller extent before any conclusions as to the failure are reached.

No serviceable correlation can be made, therefore, between grafting limits and the hybridization index as determined by Gershoy (4). In the latter work it has been shown that hybridization limits closely follow the broad morphological delineations: no crosses having been consummated between species in the major sections of the genus.

However, the section *Nominium* is divided into three distinct subsections and a few hybrids between species in different subsections are in existence. Of these, the hybrid *V. conspersa* × *papilionacea* has been discussed by Marvin (7) and by Pierce (9). *V. papilionacea* in the *Plagiostigma* subsection was used in the present work while *V. conspersa* in the *Rostellatae* subsection is closely related to *V. striata*, which formed a partially successful graft with *V. papilionacea*.

The hybrid *V. Riviniana* × *odorata*, has not yet been discussed in print. It is a vigorously growing form which is being studied in detail since it serves as a "bridging mechanism" between the subsection *Rostellatae* in which *V. Riviniana* is placed and the subsection *Uncinatae* containing *V. odorata*.[6] *V. Riviniana* is closely related to *V. striata*, which has formed successful grafts with *V. odorata*.

These data show, as might be expected, that success in grafting may parallel success in the hybridization of species to a certain extent. However, the statement that grafting limits are not correlated with the hybridization index may be made quite definite by pointing out that successful grafts have

[6] By the use of the colchicine technic the sterile F-1 hybrids have been induced to set seed. There are now in existenc, F-2, F-3, and F-4 plants which are strikingly similar in appearance. The fertile amphidiploid plants differ, characteristically, from the sterile F-1 hybrid in possessing *fewer* and somewhat shorter internodes, thicker shoots, larger and somewhat thicker leaves and flowers and, also, bigger pollen grains of which a large percentage is quite imperfect. The F-2 plants have been used in pollination with races of both parent species, thereby producing seeds of assumed back-cross origin (unpublished data). It is stated by Engler and Prantl (1) that a hybrid of this nature does not occur in the wild and, moreover, is unexpected because of the dissimilarity of the species involved.

also been obtained between species in those groups which are isolated from each other by definite hybridization barriers, i.e., the grafts *V. striata* on *V. canadensis* stock and *V. odorata* on *V. canadensis* stock have linked the *Nominium* section with the *Chamaemelanium;* the grafts *V. odorata* on *V. tricolor* stock and *V. papilionacea* on *V. tricolor* stock have linked the *Nominium* section with the *Melanium;* and the graft *V. tricolor* on *V. canadensis* stock has linked the *Melanium* section with the *Chamaemelanium.* No hybrids between species in these three sections are known to exist.

SUMMARY

An attempt has been made to determine whether or not a correlation exists between the capacity for grafting of species and the hybridization index in the genus *Viola.* Within the genus the barrier to a successful species graft centers upon a lack of adequate technic rather than upon so-called grafting incompatibilities arising in some way from the phylogenetic divergence of the species involved. This conclusion is drawn from results demonstrating potentially successful grafts in seven of the ten possible interspecific combinations among the five species chosen to represent the wide range of morphological types in the genus. There appears to be no serviceable correlation between success in hybridization of species and species grafting.

Sincere appreciation is due for the generous co-operation given by Prof. E. Matzke of Columbia University, who has made a constructive criticism of the manuscript.

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GROWTH OF FRUITS IN CATTLEYA AND ALLIED GENERA IN THE ORCHIDACEAE

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The *Cattleyeae*, a tribe of the *Orchidaceae* inhabiting the tropics and subtropics of the New World, contains the majority of the important commercial orchids. Its several genera, more than one thousand species, and some six thousand recorded hybrids form the basis of a one-hundred-million-dollar industry in this country alone. Four of the genera, *Brassavola*, *Laelia*, *Cattleya*, and *Sophronitis*, and their intergeneric hybrids indicated by combination names, such as *Brassolaeliocattleya*, contain the "Catts" of commerce.

The development of fruits in the *Cattleyeae* has been studied only by Veitch (1888) who gave the periods of time elapsing between pollination and fertilization, and between the latter and fruit maturation of *Cattleya labiata* var. *mossiae* and illustrated the fruit wall changes. In general his treatment is much like Hildebrand's (1863) and Guignard's (1886) with respect to other orchids. The value of these investigations, in addition to the periodicities listed, lies in the formation of the twofold concept that pollination functions in setting fruit and is necessary prior to fertilization.

Kirchner (1922) investigated the course of events after self pollination in orchids. He found three types of behavior, which led to sterility, based on inability of the pollen to germinate on the stigma, inability of the pollen tubes to grow down into the ovary, and production of seeds without embryos.

Duncan and Curtis (1942a) found phases of growth in both length and diameter of the fruits of *Phalaenopsis*, the phases being correlated with certain internal changes. These changes are fundamental physiological happenings, such as pollination and fertilization. They showed the inter-relationships of incompatibility-sterility factors to growth of the fruit. In *Phalaenopsis* there are three phases of growth in diameter, during which proliferation of the placentae culminating in the initiation of ovule rudiments, maturation of the macrogametophytes, and embryo growth respectively take place. The first phase of growth in length lasts through the first two phases of growth in diameter; the second, when present, takes place at the time of embryo growth. The maximum rate of macrosporogenesis occurs during a relatively quiescent period, as far as growth in diameter is concerned, between the first and second phases. Fertilization takes place when both growth in length and diameter is near a minimum.

Duncan and Curtis (1942b) found two phases of growth in diameter of fruits of *Cypripedium* and *Paphiopedilum*. These phases coincide with

macrogametophyte maturation and seed development, ovule rudiments being present at the time of pollination. A single phase of growth in length concludes at the time of fertilization.

Riley (1942) has pointed out that the radii passing through the septum and midrib of *Iris* fruits grow at relatively different rates but reversals in the rate of growth of the radii occur so that the one originally growing faster is then slower. These changes in rates, producing breaks in the growth curve, take place when the fruit shape shows evidence of changing. Fertilization apparently bears a relationship to one of the breaks.

MATERIALS AND METHODS

The genera, species, and hybrids used in the present study may be found in the following list:

Species	Hybrids
Brassavola nodosa Lindl.	× *Brassocattleya John Linford*
Cattleya amethystoglossa Lindl. & Reichb. f.	× *Bc. Jupiter*
C. bicolor Lindl.	× *Bc. Marie-Marie*
C. bowringiana Veitch	× *Bc. Yellow Hammer*
C. granulosa Lindl.	× *Brassolaeliocattleya Dorothy Fennell*
C. guttata Lindl.	× *Cattleya amabalis*
C. intermedia Graham	× *C. Brussels*
C. labiata Lindl.	× *C. Cadwalader*
C. loddigesii Lindl.	× *C. Dupreana*
C. luteola Lindl.	× *C. Enid*
C. skinneri Bateman	× *C. Kitty Wren*
C. trianaei Lindl. & Reichb f.	× *C. Mina*
Epidendrum ciliare L.	× *C. Molly*
E. cochleatum L.	× *C. Prince Shimadzu*
E. tampense Lindl.	× *Epidendrum Burtonial*
Laelia gouldiana Reichb f.	× *Laeliocattleya No. 19*
L. purpurata Lindl.	× *Lc. No. 21*
L. tenebrosa Gower	× *Lc. No. 161*
L. xanthina Lindl.	× *Lc. Canhamiana*
Leptotes bicolor Lindl.	× *Lc. Gomersal*
Schomburgkia lyonsii Lindl.	× *Lc. G. S. Ball*
	× *Lc. Schilleriana*
	× *Lc. Wellsiana*
	× *Sophrolaeliocattleya No. 519*

The studies were made at the Morris Arboretum and the botany greenhouses of the University of Pennsylvania, both in Philadelphia, and at the ranges of Dr. C. K. Schubert and the botany greenhouses of the University of Wisconsin, both in Madison.

Pollinations were cross (between clones), close (within the clone), or self (within the flower). Distant crosses were intrahybrid or interhybrid, intraspecific or interspecific. Interhybrid crosses were intrageneric or intergeneric. The number of pollinia forming the entire complement of a flower was generally used (four in *Cattleya*, six in *Leptotes*, eight in *Laelia*). There

are several exceptions caused by size of the stigmatic cavity which was sometimes too small to hold all the pollen. The most study was devoted to four forms: *Cattleya trianaei, C. bowringiana, Epidendrum tampense,* and *E. cochleatum* var. *triandrum.* Approximately 175 ovaries were measured from flower to mature fruit in all the studies.

The fruits of the *Cattleyeae* like those of *Phalaenopsis* and the *Cypripedilinae* are lined with grooves whose terminations are closely associated with the distal and proximal limits of the ovary cavity. It is not unusual, however, to find fruits whose grooves extend either somewhat down the pedicel or slightly up the beak of the ovary. Since measurements made of the pedicel and beak of the ovary do not change during fruit maturation these slight variations were disregarded and the groove length has been accepted as the length of the ovary. In later studies these limits were marked with India ink so that the distance between the same points could be measured more exactly each time. The diameter at a level about one-half the way down the ovary was marked and measurements made at this point. As the ovaries of some species enlarge the edges of certain lobes fold back, leaving an ever widening sinus in which the alternate lobes are located. The width from the enlarging edge of the wing of one lobe to that of the adjoining—in other words the width of the sinus—was measured in several series from the time of its appearance until its permanent width was reached. All measurements were made to 0.1 mm. with vernier calipers at weekly intervals. Graphs were made by plotting a moving average of the weekly percentage of total growth in length and diameter respectively.

Fruits of *Cattleya trianaei, C. bowringiana, Epidendrum tampense,* and *E. cochleatum* var. *triandrum* were removed at intervals, fixed, and sectioned for study of internal changes both in the ovary wall and ovules. When the fruits were mature the percentage of fertile seeds was determined by counting statistically significant samples of well mixed, dry seeds from each fruit. All those seeds having an opaque embryo of normal size were considered to be fertile. In some instances germination tests were used to corroborate these counts. When fruits ripened prematurely they were examined in an effort to determine the cause.

The effect of the amount of pollen on the growth of fruit was tested by varying the number of pollinia applied to the stigma. One, two, four, and

Explanation of figures 1–10

Graphs portraying the growth of fruits of ten species of the *Cattleyeae.* Growth in length is shown by solid lines; growth in diameter by dotted lines. The figures at the left of each graph represent percentage of total growth in length; those at the right percentage of total growth in diameter. FIG. 1. *Brassavola nodosa.* FIG. 2. *Epidendrum ciliare.* FIG. 3. *Laelia tenebrosa.* The last phase of growth in diameter occurs after twenty weeks and is not shown on the graph. FIG. 4. *Leptotes bicolor.* FIG. 5. *Schomburgkia lyonsii.* FIG. 6. *Epidendrum cochleatum* var. *triandrum.* FIG. 7. *Cattleya amethystoglossa.* FIG. 8. *C. bowringiana.* FIG. 9. *C. granulosa.* FIG. 10. *C. loddigesii.*

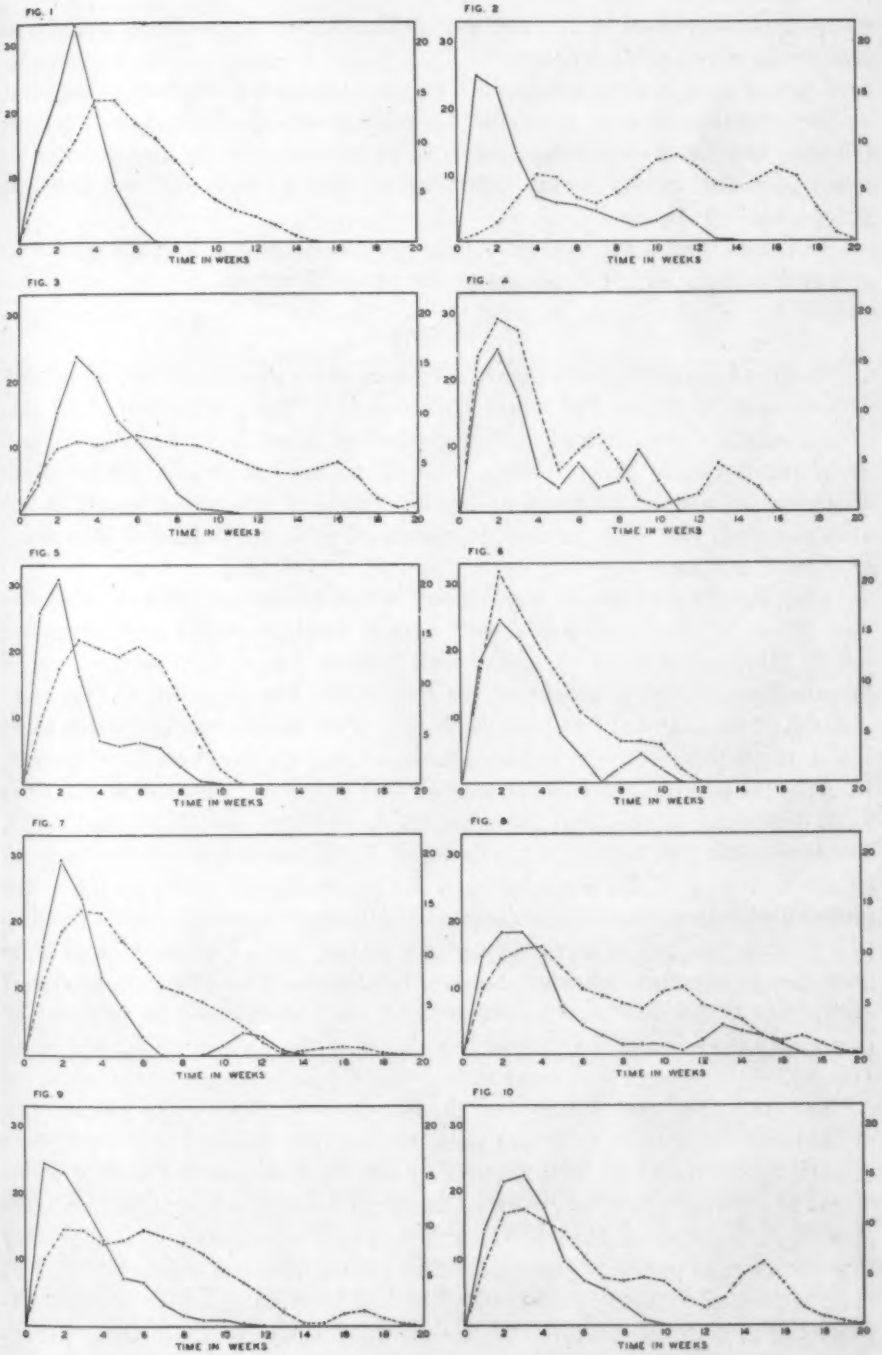


FIG. 1
FIG. 2
FIG. 3
FIG. 4
FIG. 5
FIG. 6
FIG. 7
FIG. 8
FIG. 9
FIG. 10
TIME IN WEEKS

six pollinia were used in two series of pollinations: one series on × *Cattleya Molly;* the other, on *C. trianaei.*

Light, among environmental factors, was studied with respect to its effect on the growth of fruits. A number of flowers were pollinated on *Cattleya trianaei:* one series on plants growing in an eleven-hour day and another on plants growing in day length increased to twenty hours by two 100-watt fluorescent-tube lights.

Attempts were made to simulate the pollination effects by various chemicals and to induce fruit development by artificial means.

RESULTS

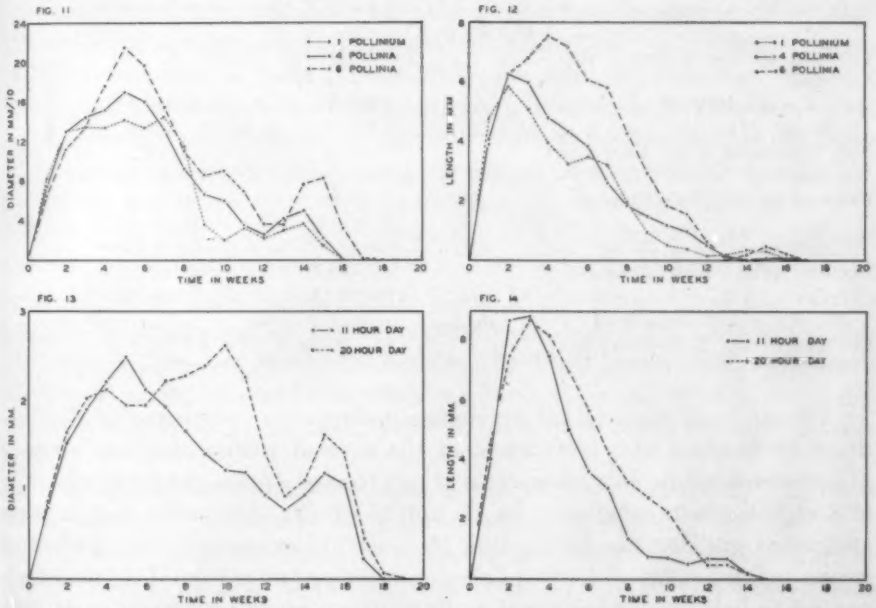
Figures 1–10 portray the growth of fruits of six genera of *Cattleyeae* and of four species within the genus *Cattleya.* All fruits represented in this group, with the exception of that illustrated by figure 5, produced seed, and are characterized by three phases of growth in diameter and one major phase of growth in length. A second and minor phase of growth in length is not always found, but when present, is associated with the third and last phase of growth in diameter.

The orchid ovary has six components which appear as lobes in cross section. Three of the components each bear a single placenta and three are sterile. Morphogenesis of the ovary wall from flower to mature fruit varies greatly from species to species of the *Cattleyeae.* For example, the six components of the ovary of *Cattleya bowringiana* are approximately of the same size in the flower and grow at about the same rate during fruit development. Flaps of the placentiferous lobes abut on each other and hide the sterile lobes until dehiscence of the fruit in certain races of *C. trianaei* (figs. 19, 23–26). The sterile lobes are hidden in the flower of *Laelia tenebrosa* but are disclosed by the widening of the sinus between the placentiferous lobes as the ovary grows. The placentiferous lobes assume a number of different forms in addition to their varying relative proportions in the fruit. Figures 15 and 17 to 20 inclusive illustrate some of these conformations. The effect of structural differences of the wall on the growth curve for diameter can be deduced by comparing the curves for *Cattleya bowringiana* (fig. 8) and *Laelia tenebrosa* (fig. 3).

The amount of pollen placed on the stigma does not alter the proportions or shape of the mature fruit but does increase the ultimate size reached in growth by the fruit (fig. 27). Figures 11 and 12 show that the weekly increments of growth in both length and diameter are increased by the application of a larger number of pollinia. Growth in length is maintained at a greater rate for a longer period of time in the first growth phase of ovaries which had been pollinated with six pollinia (fig. 12). The position and time of the interphases of growth are not affected. The peaks of the second and third phases

of growth in diameter are shifted farther apart; the duration of the interphase, in which fertilization takes place, is lengthened. The percentage (not the number) of seeds containing embryos is not significantly affected. The yields for ×*Cattleya Molly* with one pollinium is 58.8 per cent; with two, 53.3; with four, 51.8; and with six, 54.2. The yields for *C. trianaei* with one pollinium is 51.2 per cent; with four, 54.0; and with six, 61.0. This series seems to indicate an upward trend with increasing numbers of pollinia but any possible significance is nullified by the results with ×*C. Molly*.

Increasing the effective day length from eleven to twenty hours brings about the maintenance of growth in length and diameter at a higher rate



FIGS. 11–14. Graphs illustrating growth of fruits of *Cattleya trianaei*. FIG. 11. Increment curves of growth in diameter for three fruits of a late blooming form, each with a different number of pollinia. FIG. 12. Increment curves of growth in length for the same fruits as in figure 11. FIG 13. Increment curves of growth in diameter for fruits of two plants of an early blooming form, each exposed to different day lengths. FIG. 14. Increment curves of growth in length for the same fruits as in figure 13.

over a longer period of time (figs. 13, 14). This is particularly true with respect to diameter; however, growth in diameter in the twenty-hour day never reaches the peak rate that is reached under the eleven-hour day. The period of slower growth between the first and second phases of growth in diameter it obscured. Neither the period of time necessary for the maturation of the fruit nor the percentage of seeds possessing embryos in a fruit is altered by the increased day length. The percentages were 55.2 for a fruit from the eleven-hour day and 52.9 from the twenty-hour day.

The following list is made up of selected crosses of various kinds. Low percentage of viable seeds is reflected to a limited extent in curves representing increments of growth in diameter.

PERCENTAGE OF SEEDS POSSESSING EMBRYOS

	Per cent
Cattleya species	
C. trianaei, cross pollinated (8)	37.6–61.4
C. trianaei, selfed (1)	0.0
C. bowringiana, cross pollinated (4)	66.7–78.3
Laelia species	
L. tenebrosa, cross pollinated (2)	48.3–64.8
Cattleya species X *Laelia* species	
C. bowringiana X *L. tenebrosa* (2)	41.7–62.1
L. tenebrosa X *C. bowringiana* (2)	6.2–19.7
Cattleya species X *Cattleya* hybrid	
C. loddigesii X × *C. Molly* (1)	63.6
Cattleya hybrid X *Cattleya* species	
× *C. Molly* X *C. labiata* (1)	64.1
Cattleya hybrid X *Cattleya* hybrid	
× *C. Molly*, cross pollinated (4)	51.8–54.2
× *C. Molly*, selfed (1)	0.0
Laeliocattleya hybrid X *Laeliocattleya* hybrid	
× *Lc. No. 21*, close pollinated (2)	17.1–17.2
× *Lc. No. 19*, cross pollinated (1)	28.0
Laeliocattleya hybrid X *Cattleya* hybrid	
× *Lc. No. 21* X × *C. Molly* (1)	11.1
× *Lc. No. 21* X × *C. Kitty Wren* (1)	36.2
Laelia species X *Cattleya* hybrid	
L. tenebrosa X × *C. Molly* (1)	87.2
Cattleya hybrid X *Laelia* species	
× *C. Molly* X *L. tenebrosa* (1)	66.6
Cattleya hybrid X *Laeliocattleya* hybrid	
× *C. Molly* X × *Lc. No. 19* (1)	64.8

The study of the internal development shows that placentae of *Epidendrum cochleatum* var. *triandrum*, at the time of pollination, has already undergone some proliferation. This is not true of *Laelia xanthina* (fig. 17) or *Cattleya amethystoglossa*. In *C. granulosa* (fig. 18) there is a certain amount of proliferation by the time the perianth expands but the placental ridges are still reflexed. *Cattleya amethystoglossa* has placental ridges which project radially toward the center of the ovary cavity; however, little proliferation has taken place by the time of anthesis. One week after pollination the ovary of *E. cochleatum* var. *triandrum* has enlarged considerably through periclinal elongation of the inner cell layers of the ovary wall. This, together with the enlargement of outer cells of the ovary wall, allows a considerable increase in the amount of ovary cavity into which the placentae have extended through enlargement of their constituent cells. Only scattered nuclear and cell divisions were seen in the ovary wall, those present being in the vicinity of the veins, the hypodermal layer of the placentiferous lobes, and the regions where dehiscence will take place at maturity. Greatly enlarged scattered cells contain either raphides or elaioplasts, both having been present in cells of the ovary of the unpollinated flower. There seems to have been a general increase in the number of cell layers in the portion of the

ovary wall adjoining the cavity but insufficient cell divisions have been seen to account for the approximately five extra cell layers of the wall. It is possible that these took place as in *Phalaenopsis* very shortly after pollination—within two or three days. The proliferations of the placentae in the week-old fruit show some scattered nuclear and cell divisions but in general the tips of the ramifications have assumed the shape of an ovule rudiment. In some cases the hypodermal cell has enlarged somewhat, and has the appearance of a primary archesporial cell.

At the end of the second week groups of pollen tubes are found growing along the sides of the placentae. The ovules show the beginnings of the first integument and a curvature. There are a considerable number of division figures in the nucellus and beginning integuments. One week later the macrospore mother-cells of most of the ovules are entering reduction division, although a few ovules contain four-nucleate macrogametophytes. At the close of the fourth week a few ovules are ready for fertilization, but the majority still contain macrospore mother cells in some stage of reduction division.

By the end of the fifth week, at the time of a second phase of growth in diameter which is very closely associated with the first (fig. 6), a greater portion of the ovules have assumed mature form and shape. During the ensuing two weeks fertilization takes place. At the conclusion of seven weeks most of the ovules contain small embryos. At the end of the ninth week, during the third phase of growth in diameter (fig. 6), the embryos have enlarged greatly, their suspensors have grown out through the micropyle and reached the surface of the placentae. The seed coats have taken on their mature texture.

The intervals at which the ovaries of other forms reach the critical points of meiosis and fertilization vary considerably. In many of the one-leaved species of *Cattleya* the first growth period intervening between pollination and reduction division in the ovules is about seventy days. Figure 28 is a photograph of the bundle of pollen tubes dissected out of a fruit of *C. trianaei* just prior to fertilization. The column and beak of the ovary which is left attached at the upper end gives an indication of the relatively large proportion of pollen tubes to ovary.

The period necessary for ripening of the fruit and the natural dissemination of the seeds varies from three months for *Epidendrum cochleatum* var. *triandrum* to fifteen for *Cattleya trianaei* under greenhouse conditions in Wisconsin.

Induction of parthenocarpy by artificial means was attempted in a series of exploratory experiments conducted over a three-year period. Fourteen species in the following genera, of which only *Laelia* and *Cattleya* belong in the *Cattleyeae*, were used as experimental material: *Cattleya, Cyrtopodium, Laelia, Miltonia, Oncidium, Paphiopedilum, Phalaenopsis, Vanda,* and *Zygo-*

petalum. Substances employed were: indole-acetic, indole-propionic, indole-butyric, and naphthalene acetic acids; Gonadogen; water and alcohol extracts of both ungerminated pollinia and pollen tubes. These agencies were tried in different concentrations in water solution, in glycerine solution, in lanolin emulsion, and as dry crystals (where possible). They were applied externally by spraying or painting, internally by injection into either the ovary cavity or the nectary, or were introduced into the stigmatic depression in cotton pellets or dry agar blocks in simulation of natural pollination.

The heteroauxins and the pollen extracts brought about wilting of the perianth in all species, except in *Zygopetalum* whose perianth segments do not wilt even after pollination. The stigma changes associated with pollination in *Phalaenopsis* were duplicated by 0.1 per cent indole-acetic acid in lanolin. The most efficient method of application was the dry agar block technique. Apparently the absorption of water by the agar influenced the water relations of the perianth in the same manner as the natural pollinia. Initial elongation of the ovary was noted in *Cattleya* (maximum elongation was 7 mm. in *C. trianaei*). In no instance did any of the treatments on any form result in fruit formation.[1]

DISCUSSION

Comparison of the growth curves of the various members of the *Cattleyeae* included in this report reveal considerable variation in length of time elapsing between pollination, meiosis, fertilization, and maturation of the fruit respectively. There is a further variation in the relative magnitude and proportions of the various parts of growth curves erected on weekly increments of growth. *Epidendrum ciliare* var. *latifolia* (fig. 2) and *E. cochleatum* var. *triandrum* (fig. 6), for example, differ greatly in the duration of the first phase of growth in diameter, the former having a considerably extended first phase. *E. cochleatum* var. *triandrum* has a second phase of growth in diameter which is greatly telescoped by the first but clearly distinct from the third. In general, however, there are three phases of growth in diameter and one, sometimes two, of growth in length of fruits of the *Cattleyeae* studied. The phases of growth in diameter coincided respectively with proliferation of the placentae and initiation of ovule rudiments, growth of the ovule and maturation of the macrogametophyte, and growth of the embryo and maturation of the seed. The first phase of growth in length is generally completed before

[1] Hubert and Maton (Parthenocarpie en groeistof. Natuurwetenschap. Tidjschr. 21: 339–348. 1940.) have reported the production of parthenocarpic fruits of considerable size induced by the application of crystals of naphthaleneacetic acid on the stigmas of a *Cymbidium* hybrid and *Oncidium longipes*. The bearing of our own results on this report is obscure. The publication has not been available to the authors.

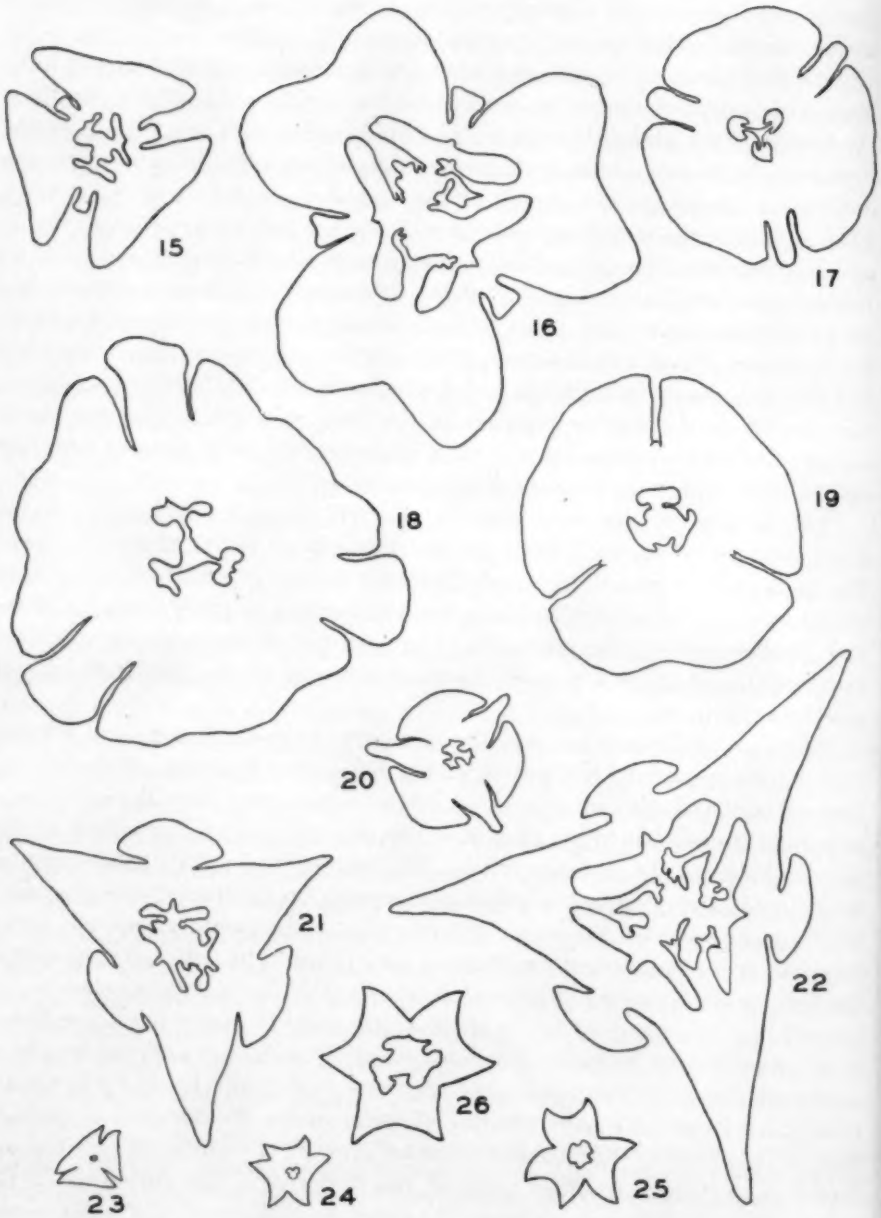
Since this paper has gone to press we have had some success in setting parthenocarpic fruit on *Zygopetalum mackayii* with naphthoxyacetic acid and naphthalene acetamide.

the end of the second phase of growth in diameter. At the conclusion of the first phase of growth in diameter meiosis is taking place. At the conclusion of the second in diameter fertilization occurs.

The first phase of growth in diameter is sometimes modified by the initiation and growth of a sinus, as in *Cattleya trianaei* (figs. 19, 23–26), between the wings of the placentiferous lobes. It is possible that telescoping of the first two phases of growth in diameter in *Epidendrum cochleatum* var. *triandrum* is caused by the growth of the singular projection of the placentiferous lobe at the time of meiosis (figs. 20–22). *Epidendrum ciliare* (fig. 2) and *E. tampense* (figs. 15 and 16), which do not possess such projections, do possess clear cut first and second phases of growth in diameter. Shortening the period devoted to each phase (climate would shorten the growing period) tends to run the phases together. *Epidendrum cochleatum* var. *triandrum* has the shortest ripening period of any member of the *Cattleyeae* studied; it is native to the northern shores of the Gulf of Mexico. The phases, of course, could be portrayed graphically more readily by the use of four-day rather than weekly intervals of measurement.

The growth of the fruit and its relation to internal changes in the *Cattleyeae* agree precisely with the development of fruits of *Phalaenopsis*. The peak rate of growth in length generally occurs at about the same time as the peak rate of growth in diameter; this feature of the *Cattleyeae* is unlike *Phalaenopsis* whose peak rate of growth in length occurs between the first two phases of growth in diameter at the time of the maximum rate of macrosporogenesis.

The chief difference between the members of the *Cattleyeae* and *Phalaenopsis* with respect to pollination effects lies in the behavior of the stigma. The edges of the stigmatic cavity of *Phalaenopsis* grow over the cavity and imprison the pollinia. This growth is brought about by enlargement of the cells making up the tissue involved. The stigma in the *Cattleyeae* remains unchanged after pollination with the exception that at times more stigmatic fluid may be secreted. Since this difference is one that mainly concerns water relations it is possible that an effect on growth could be detected. The initial lag for one or two weeks in growth in length of fruits of *Phalaenopsis* is not detected in the growth of fruits of the *Cattleyeae*. If such a lag is present it is of considerably reduced duration. A rapid initial growth in length is characteristic of the *Cattleyeae*, the only exception found being *Epidendrum ciliare*. An immediate redistribution of water under the stimulus of pollination is indicated by the simultaneous collapse of the perianth and the enlargement of tissue in other parts of the flower—i.e., the enfolding of the stigma edges in *Phalaenopsis* and the elongation of ovary wall cells in the *Cattleyeae*. The application of heteroauxins brings about these respective effects.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Growth in diameter of the ovaries of *Cypripedium* and *Paphiopedilum* is characterized by two growth phases: during the first, meiosis and development of the macrogametophyte occur; during the second, seed maturation. Fertilization takes place in the period of little or no growth between these two phases. Growth in length has concluded by this time. At the time of pollination the ovules of *Cypripedium* and *Paphiopedilum* are present and consist of a nucellus containing a distinct archesporial cell and the beginnings of the inner integument. The two growth phases in diameter in the *Cypripedilinae* are homologous to the latter two phases of growth in diameter in the fruits of the *Cattleyeae*.

Duncan and Curtis (1942b) suggested that three types of fruit development could be distinguished by the number of phases of growth in diameter, the number present being correlated with the stage of development of the ovary at the time of pollination. If the flower opens when the placentae have not undergone proliferation, three phases of growth in diameter of the ovary are present; if the flower opens when the ovule rudiments are present, two; and if the flower opens when the ovules are ready for fertilization, one. The first type should be modified, according to observations in the *Cattleyeae*, to include flowers whose ovaries contain at anthesis placentae which have proliferated to a degree but have not undergone their entire proliferation.

The differences in the development of the component parts of the fruit wall, such as the initiation and growth of wings or reflexing of the edges of the placentiferous lobes, affect the shape of the curve representing the first growth phase in diameter. Since these structural changes vary from species to species of the *Cattleyeae* the curves representing growth in diameter of a species may possess a certain amount of individuality. The growth of the sinus in *Laelia tenebrosa* brings about a trimodal appearance of the first growth phase in diameter as the initiation, waxing, and waning of the growth leading to the widening of the sinus contributes to or decreases the rate of growth in diameter. Such fluctuations are emphasized by the presence of smaller amounts of pollen on the stigma as is demonstrated in *Cattleya trianaei* (fig. 11). If the six components undergo about the same develop-

Explanation of figures 15–26

Diagrams of cross-sections of orchid ovaries. FIG. 15. *Epidendrum tampense*. Cross-section of ovary of an unpollinated flower. FIG. 16. *E. tampense*. Cross-section of ovary containing seed. FIG. 17. *Laelia xanthina*. Cross-section of ovary of unpollinated flower. FIG. 18. *Cattleya granulosa*. Cross-section of ovary of unpollinated flower. FIG. 19. *C. trianaei*. Cross-section of ovary of unpollinated flower. FIG. 20. *Epidendrum cochleatum* var. *triandrum*. Cross-section of ovary of unpollinated flower. FIG. 21. *E. cochleatum* var. *triandrum*. Cross-section of ovary two weeks after pollination. FIG. 22. *E. cochleatum* var. *triandrum*. Cross-section of ovary three weeks after pollination. FIGS. 15–22. × 8. FIGS. 23–26. *Cattleya trianaei*. Cross-sections of ovaries showing the folding back of the wings of the placentiferous lobes at three, four, nine, and thirteen weeks after pollination. One-half natural size.

ment or if the proportion of sterile to placentiferous components remains about the same during the course of development, the growth curve is little affected, an example being *Cattleya bowringiana*. This is true of *Phalaenopsis*.

Since an increase in the number of pollinia applied to the stigma merely increases the size of the fruit, it may appear to be brought about by the increase in dosage of the growth regulatory compound or compounds which they contain. Extracts of the pollen, even though they do not give rise to parthenocarpic fruits, produce the same initial effects as the pollen itself. The substance responsible for fruit set may be produced by germinating pollen grains. Injections with water extracts of pollen tubes from pollen germinated on agar or on fluid of stigmas from excised columns, however,

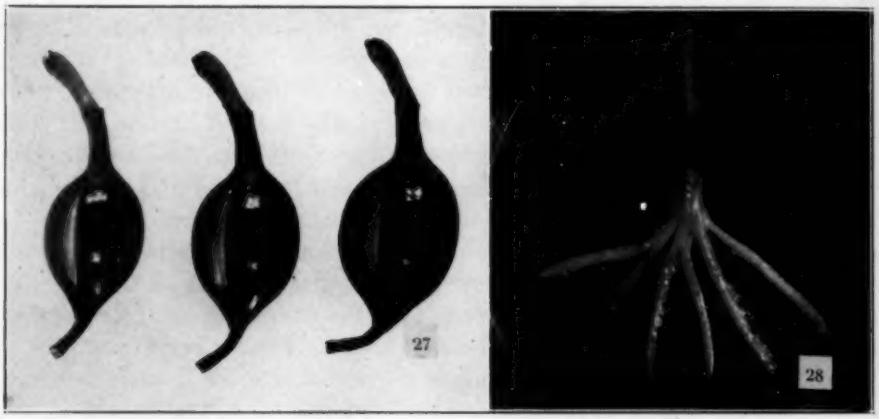

FIG. 27. Mature fruits of *Cattleya trianaei*. The smallest fruit received one pollinium, the intermediate, four pollinia, and the largest, six pollinia. The data for figures 11 and 12 were obtained from these fruits.

FIG. 28. Bundle of pollen tubes dissected from a fruit of *Cattleya trianaei* just before fertilization. The beak of the ovary is left attached to indicate the relative size of the pollen tubes.

gave negative results. This suggests that some relationship between stigmatic or stylar tissue and pollen tubes may be necessary—that one may activate the production of the growth regulatory compound in the other. These findings are in line with the conception that the entrance of pollen tubes into the ovary cavity is necessary for fruit set.

Certain of the reagents used in the work with orchids, injected into or sprayed on flowers of such plants as *Carica papaya* and *Lycopersicon esculentum*, caused ripening of parthenocarpic fruits. Although our experiments are by no means exhaustive, it would appear that the substances responsible for fruit set in the *Orchidaceae* are different from, or at least are not acti-

vated by, the same substances that Gustafson (1938) found to be effective in a wide range of dicotyledonous genera.

Figure 5 illustrates the fact that a fruit set with incompatible pollen never passes through all the growth phases of the fruit but ripens prematurely. This close mating belongs to the category of incompatibility in which germination of the pollen and entrance of the pollen tubes into the ovary take place but fertilization fails, the apparent cause being the inability of the pollen tubes to grow out to any great extent over the extremities of the placentae. The ovules do not become seed-like. Two earlier occurring types of incompatibility, the inability of the pollen to germinate on the stigma or of the pollen tubes to grown down through the stylar tissue, fail to do more than hold the ovary on the inflorescence for longer than a few weeks, abscission taking place at the base of the pedicel. The fruit of ×*Cattleya Molly*, given in the list of "percentage of seeds possessing embryos" as zero per cent, contained ovules whose integuments were of mature size and texture. The curve representing growth in diameter of this fruit suggests a third growth phase. It is possible that the cause of this type of sterility lies after fertilization. The third phase of growth in diameter in corresponding fruits of *Phalaenopsis* was not detected, and this type of sterility was grouped with those in which the pollen tubes fail to grow out over the placentae and the fruit ripens prematurely. These, too, may have been brought about by post-fertilization failures. Fruits affected by this type of sterility ripen somewhat prematurely. The difficulty in assigning the cause in such chance cases lies in the fact that by the time the fruit has ripened the tips of the pollen tubes have disintegrated.

The list of percentage of seeds possessing embryos brings out several facts that should be of interest to the practical breeder of orchids. As far as general breeding work is concerned they offer nothing new. Mating members of a species with other members of the same species seldom results in exceptionally high seed set. The section of the genus *Cattleya* to which *C. bowringiana* belongs, the *Diphyllae*, yields the highest counts. Reciprocals by no means give equally good results. The cross between *Laelia tenebrosa* and *Cattleya bowringiana* is an example. The cause of the inequality between the reciprocals may lie in the great difference in the length of the beak of the ovary in *Laelia tenebrosa* and *Cattleya bowringiana* and corresponding differences in the length of their pollen tubes.

×*Cattleya Molly* is an extremely heterozygous hybrid. It includes five species in its ancestry, no one of which is duplicated. When self- or close-pollinated the percentage of seeds containing embryos is low (0.0 per cent and 9.6 per cent respectively). Back crossing on the species, *C. labiata*, does not increase the yield over that of wide crosses with a member of another genus, *Laelia tenebrosa*, or with intergeneric hybrids such as ×*Laeliocattleya*

No. 19 although it does increase the yield over crosses between different plants of ×*Cattleya Molly*. It is interesting to note that the fertility of ×*C. Molly* is about the same as that of *C. trianaei*, a species.

The complex intergeneric hybrids *Laeliocattleya No. 19* and *No. 21* show more sterility than ×*Cattleya Molly* when close pollinated or when individual plants of the respective hybrids are mated among themselves. There is, however, a wide range of fertility when used as either a pollen or a female parent in a wide cross. Since there is a certain amount of back crossing on species in their ancestry the individual plants of each hybrid may behave quite differently. This may explain the wide range of values. ×*Laeliocattleya No. 21* shows more sterility than ×*Lc. No. 19*. The intergeneric hybrids between species of *Brassavola* and *Cattleya*, like those between *Laelia* and *Cattleya*, have considerably reduced fertility. The percentage of seeds possessing embryos was not determined for hybrids whose ancestry includes three or four genera.

It is perhaps not surprising that the orchids, which have evolved such a high degree of specialization to insure cross pollination, should give higher percentages of good seeds when matings are between clones. The cleistogamous orchids, however, according to Kirchner (1922) are highly fertile. It is surprising, however, that crosses between members of different species or even genera should give better results than selfing. This emphasizes the fact that the barriers between species and genera in the *Cattleyeae* are slight. During the past sixty years of breeding only two groups, relatively incompatible with other members of this tribe, have been found. They are the reed-like section of *Epidendrum* and the Mexican section of *Laelia*.

SUMMARY

The type of fruit development first described in *Phalaenopsis* is found in the *Cattleyeae*.

The basic trimodal growth curve may be modified by the specific conformations of the fruit wall and the relative growth rate of the components.

The amount of pollen affects the ultimate size but not the proportions of the fruit.

Increasing the day length contributes toward a larger fruit.

Neither light nor a variant amount of pollen, within reasonable limits, significantly affect the percentage of seeds possessing embryos.

Three incompatibility types and probably a type of sterility caused by a post-fertilization failure are described.

Compilation of the percentages of seed possessing embryos indicates that selfing lowers the yield and wide crosses (between genera or species) often give as good a yield as crosses within a species. Extreme hybridity points

out the lack of well defined barriers to breeding between species and genera; this is correlated with their systematic classification.

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BULLETIN OF THE TORREY BOTANICAL CLUB
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A MYCORRHIZOME FROM THE CARBONIFEROUS OF ILLINOIS[1]

HENRY N. ANDREWS AND L. WAYNE LENZ

In a recent description of a petrified Coenopterid fern stem from the middle Pennsylvanian of Illinois the senior author mentioned the presence of abundant mycelium in the cortical cells and suggested that they might be of a mycorrhizal nature (Andrews 1942). A detailed study of this associated fungus leaves little doubt that that supposition was correct and the abundance of the fungus, its exceptionally fine preservation, and the diversity of the morphology presented renders a description desirable.

Fossil mycelium is not uncommon. It has been reported in Carboniferous plant remains and is frequently encountered in Cretaceous and Tertiary lignites. Unfortunately the hyphae rarely display significant diagnostic characters. The only records of fossil mycelium that may with little question be considered mycorrhizal are those described by Weiss (1904) and Osborn (1909) from the Coal Measures of England. The fungus described by Weiss was found in an unidentified root (or rhizome?) and was given the name *Mycorhizonium*. It may be well to point out that that name was used as a monomial and consequently is not valid as a generic designation. It seems highly probable, however, that Weiss' interpretation of the mycelium as mycorrhizal is correct and his contribution is of significance since it shows that such an association existed in Carboniferous times. The organism described below fully confirms this and adds appreciably to our knowledge of the morphology of the Paleozoic forms. A glance at the figures may, moreover, eliminate any doubt that botanists may harbor about the quality of preservation to be found in American coal balls.

The mycorrhiza reported by Osborn was found in the cortical cells of *Amyelon radicans*. He observed "large knob-like growths with thickened walls that are apparently terminal on the hyphae, and are about .05 mm. in diameter. These I take to be a form of resting body which the fungus forms to carry it over the period during which the cortex will be sloughed . . ." (p. 607). These structures are possibly comparable to supposed vesicles described here in our Illinois specimens.

The fungus occurs in the cortical cells of a fern stem, possibly a rhizome, described as *Scleropteris illinoiensis* (Andrews 1942). The mycelium is found within the host cells throughout the cortex although it is somewhat more abundant in the middle and inner regions. Hyphae were also found in

[1] A study aided by a grant from the Penrose Fund of the American Philosophical Society.

the tracheids of the stele although they do not assume the typical mycorrhizal form in these cells.

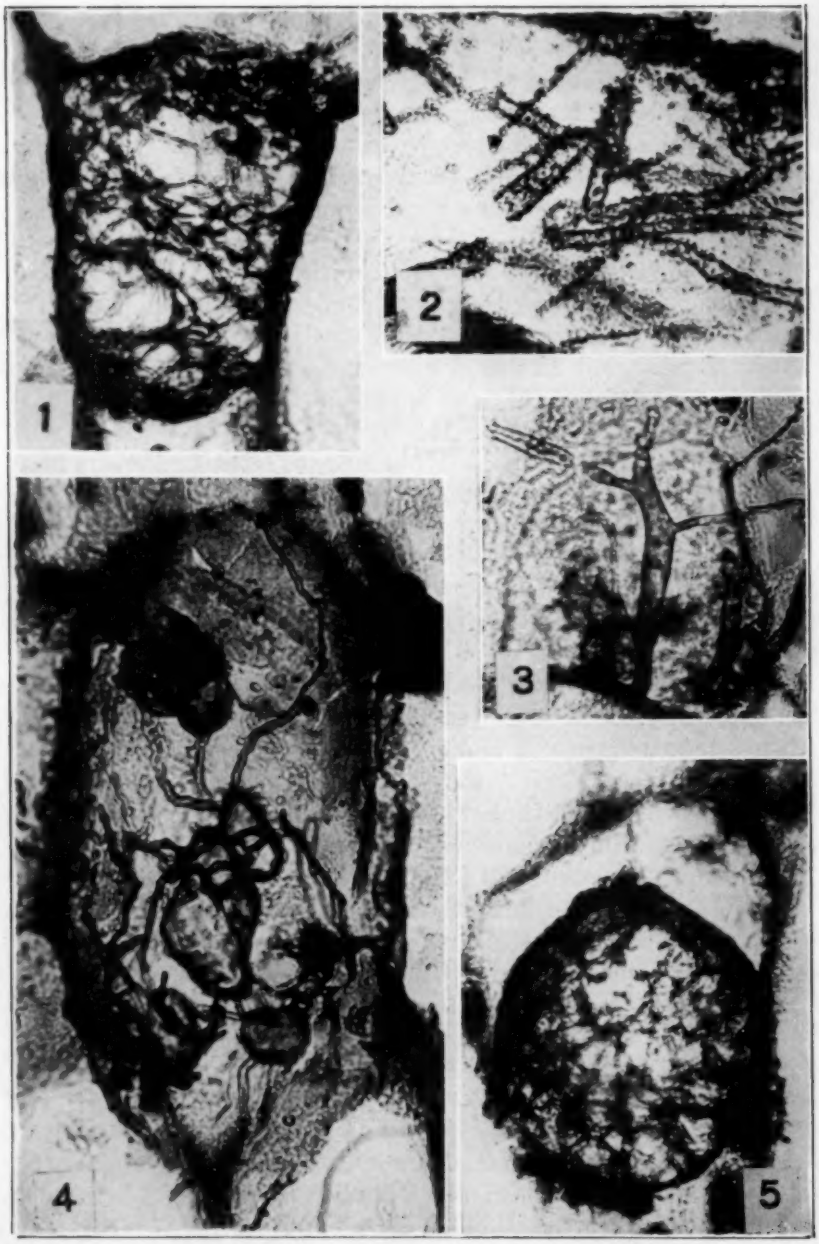
The proper application of the term mycorrhiza may be considered at this point in view of the anatomical nature of the host organ. As stated above the latter is a stem consequently the word mycorrhiza is hardly appropriate. In view of the fact that the fungus is infecting or living symbiotically with a stem or rhizome the term mycorrhizome seems more suitable and will be used in our description.

A glance at the figures reveals the diversity of the hyphal morphology. Whether or not all of this mycelium belongs to the same species of fungus cannot be stated positively. However, all of the figures are taken from the same host specimen and all within a few millimeters of each other. Although most of the mycelium seems to be intracellular, appearing typically endotrophic, there is some evidence that it may be intercellular as well. In figure 6 it appears as though some hyphae may be intercellular with branches or branching haustoria penetrating into the cell lumen. The dividing line between ectotrophic mycorrhizae or mycorrhizomata is probably not so sharp as was once supposed. Indeed Young (1940) states that "The difference between the two types of mycorrhizae is now known to be one of degree rather than of quality. They merge into one another and many intermediate forms are known."

A considerable number of host cells contain a very dense aggregation of mycelium (figs. 1, 9). It was the characteristic appearance of these cells that first attracted our attention and suggested their mycorrhizomatal nature. The hyphae average about 1 μ or less in diameter and are more or less uniformly distributed about the periphery of the cell lumen. There is some similarity between this organization and the Hartig net of the mycorrhizae of living plants. However, the slender much branched hyphae shown in these figures are clearly intracellular thus the comparison is only a superficial one. In many of the host cells infected in this way the mycellium tends to assume a nearly spherical form (fig. 5). Not only does it aggregate into compact spherical masses but as this continues the hyphae seem to become irregularly fused together, losing their identity as individual strands. This apparent fusion continues until in some cells the entire mycelial body appears as a nearly uniform amber-colored sphere.

It is perhaps most probable that this has resulted from plasmolysis of the entire contents of the host cell, but there is the possibility that this transition is the result of the digestion of the fungus by the host cells, which thus retain a symbiotic balance between host and fungus as reported in the Orchidaceae (Rayner 1927, p. 67).

Many of the cortical cells of the host harbor but a few strands of branching mycelium. These usually consist of rather large uniform hyphae bearing



FIGS. 1–5. A fossil mycorrhizome from Illinois. FIG. 1. WCB90B–S13. FIG. 2. WCB90B–S34. FIG. 3. WCB90B–S34. FIG. 4. WCB90B–S34. FIG. 5. WCB90B–S13. All figures × 500.

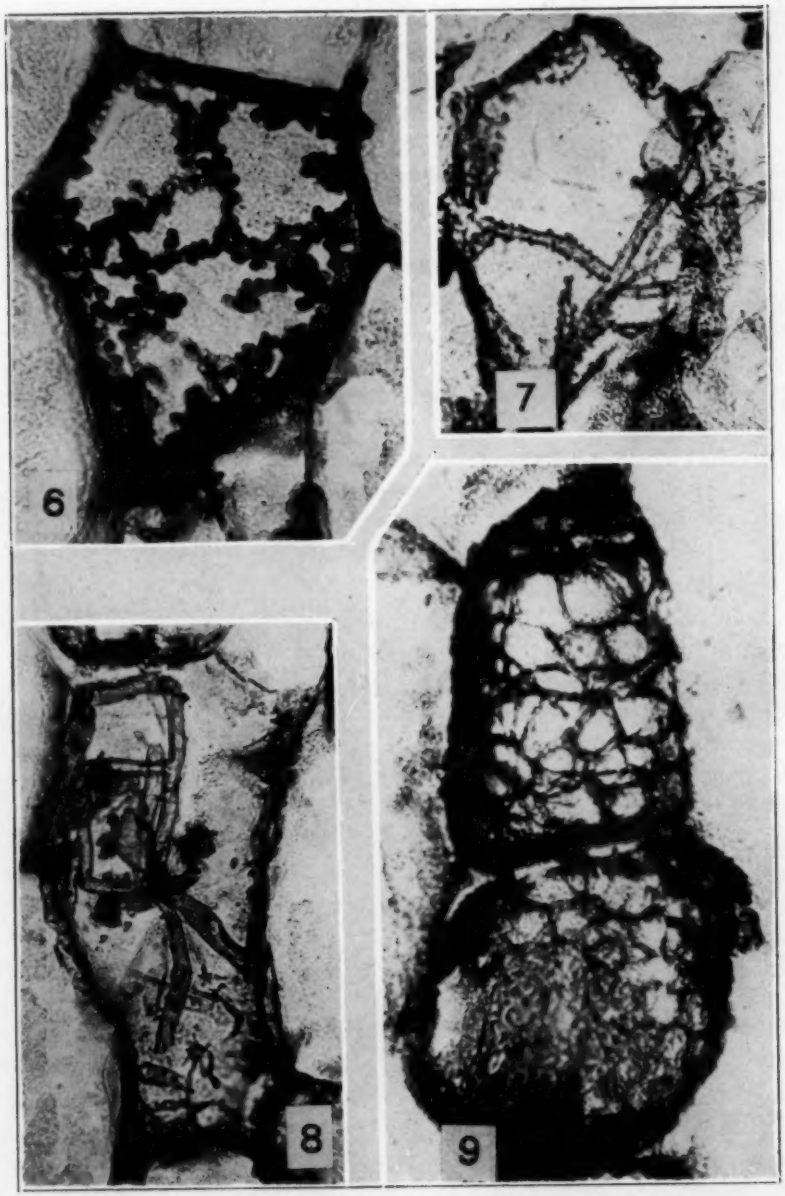
noticeably smaller branches (fig. 3). Filaments such as those shown in figure 8 average 6 μ in diameter while those in figure 4 are about 2 μ wide. The comparatively large size of some of the hyphae is clearly shown in figures 2 and 3, both of which are magnified 500 times, while those in figure 8 are magnified but 350 times. We have occasionally observed cross walls in these larger hyphae but they are rare and in most cases there is no evidence of septation.

Some of the other distinctive fungal features may now be considered. The type of mycelial development illustrated in figure 6 occurs in many cortical cells. It has been suggested that these knotty outpocketings of the hyphae may represent an initial stage in the development of the peripheral net of figures 1 and 9. This is probably not the case since the mycelium of infected cells such as those shown in figures 1 and 6 and always quite distinct. We have not observed any phase that might be considered intermediate between the two. Furthermore the hyphae of figure 6 are notably of greater diameter than the hyphae of figures 1, 5, and 9. It may be that figure 6 represents a specialized haustorial type of hyphae.

In a few of the cortical cells (fig. 4) there may be noted a number of larger bodies varying from 15 to 33 μ in diameter. Although we have not been able to observe the actual point of attachment it is probable that these bodies have had their origin from the swollen tip of a hyphal branch and we tentatively consider them to be comparable to the vesicles described in recent mycorrhizae. In a few of the cortical cells the larger hyphae exhibit a peculiar warty appearance (figs. 2, 7). It is difficult to be certain whether this appearance is due to minute protrusions of the hyphae or simply an aggregation of granules. Janse (1897, p. 65) has described a somewhat similar appearing accumulation of starch granules about the nuclei in *Ophioderma*. However in our material the "granules" appear to exist in organic connection with the large hyphae and we are inclined to favor the former interpretation.

In view of the wide diversity of the hyphal morphology (cf. figs. 2, 4, 6, 9) it seems likely that more than one species of fungus is represented. Furthermore our assumption that the association represented is actually mycorrhizomatal and not simply pathological is based on a general comparison with living forms. The dense tangles of mycelium shown in figures 1 and 9 compare closely with many described living mycorrhizae and if the more or less ovoid bodies shown in figure 4 are true vesicles this adds considerable weight to their supposed mycorrhizomatal nature. It has been suggested that the fungus shown in figure 6 is similar to that found in some pines.

Although the previously mentioned works of Weiss and Osborn as well as the present contribution must be considered only as introductory, the



FIGS. 6–8. A fossil mycorrhizome from Illinois. FIG. 6. WCB90B–S30. FIG. 7. WCB90B–S30. FIG. 8. WCB90B–S34. FIG. 9. WCB90B–S13. Figures 6 and 9, × 500; figures 7 and 8, × 350.

evidence accumulated to date rather strongly evinces the existence of this type of association as early as Pennsylvanian times. In view of the already proven important part that mycorrhizae or mycorrhizomata play in so many living plants it is to be hoped that the fossil record may continue to reveal significant fragments of their ancestral development.

We are especially indebted to Dr. Arthur P. Kelley and Prof. P. R. Gast for assistance in interpreting the fungi described here, although those authorities are in no wise responsible for the views that we have expressed.

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SOME FOSSIL FUNGI FROM MINNESOTA[1]

C. O. ROSENDAHL

Several years ago in the course of studying a collection of fruits, seeds, and other plant remains of Pleistocene age obtained from a deep well situated near the village of Bronson in southeastern Kittson County, Minnesota, I discovered a number of small clusters of brown sporangia-like bodies on branching thick-walled hyphae (fig. 1). The vegetable mixture in which they occurred had been recovered from clods of silt brought up in the slush-bucket in the process of drilling the well and had been freed of silt by washing through a fine-meshed wire screen. Whether these clusters had been intimately associated with plant fragments or occurred scattered through the silt matrix was not ascertained. In no case, however, were they found in organic connection with fragments of roots, leaves, bark, or other vegetable material. Superficially the organism resembled a mold, but the thick-walled, irregular hyphae and the aberrant "sporangia" clearly indicated that it did not belong in the Mucorineae. After several unsuccessful attempts to get the organism identified or to obtain a clue to its possible relationship it was reluctantly put aside as probably indeterminable. Most likely it would have remained permanently shelved except for the kindly interest of Dr. G. Bisby, of the Imperial Mycological Institute of Kew, who had seen a preparation of the material. He called my attention to a paper by Dr. E. J. Butler (1939) entitled "On the occurrence and systematic position of the vesicular-arbuscular type of mycorrhizal fungi," which had been published a few weeks earlier in the Transactions of the British Mycological Society.

In addition to his treatment of recent forms of the fungus (*Rhizophagus*) Dr. Butler also described and figured a representative of the group obtained from glacial clays underlying muskegs in the neighborhood of Edmonton, Alberta. A comparison of the Bronson well organism with Dr. Butler's illustrations, further supported by the descriptive details, soon convinced me that it belonged to the same group as the one from the Alberta bogs.[2]

The Bronson well fungus was found at a depth of 88–89 feet. The vegetable layer in which it occurred appears to have been a forest floor, perhaps of a spruce-tamarack bog, judging from the abundant remains of these trees, together with northern bog mosses. That the site had passed through the successional stages from open water to bog is evidenced by the occurrence

[1] This investigation has been aided in part by a grant from the Graduate School of the University of Minnesota.

[2] In subsequent correspondence with Dr. Butler he has confirmed this view after seeing a photograph of the Minnesota material.

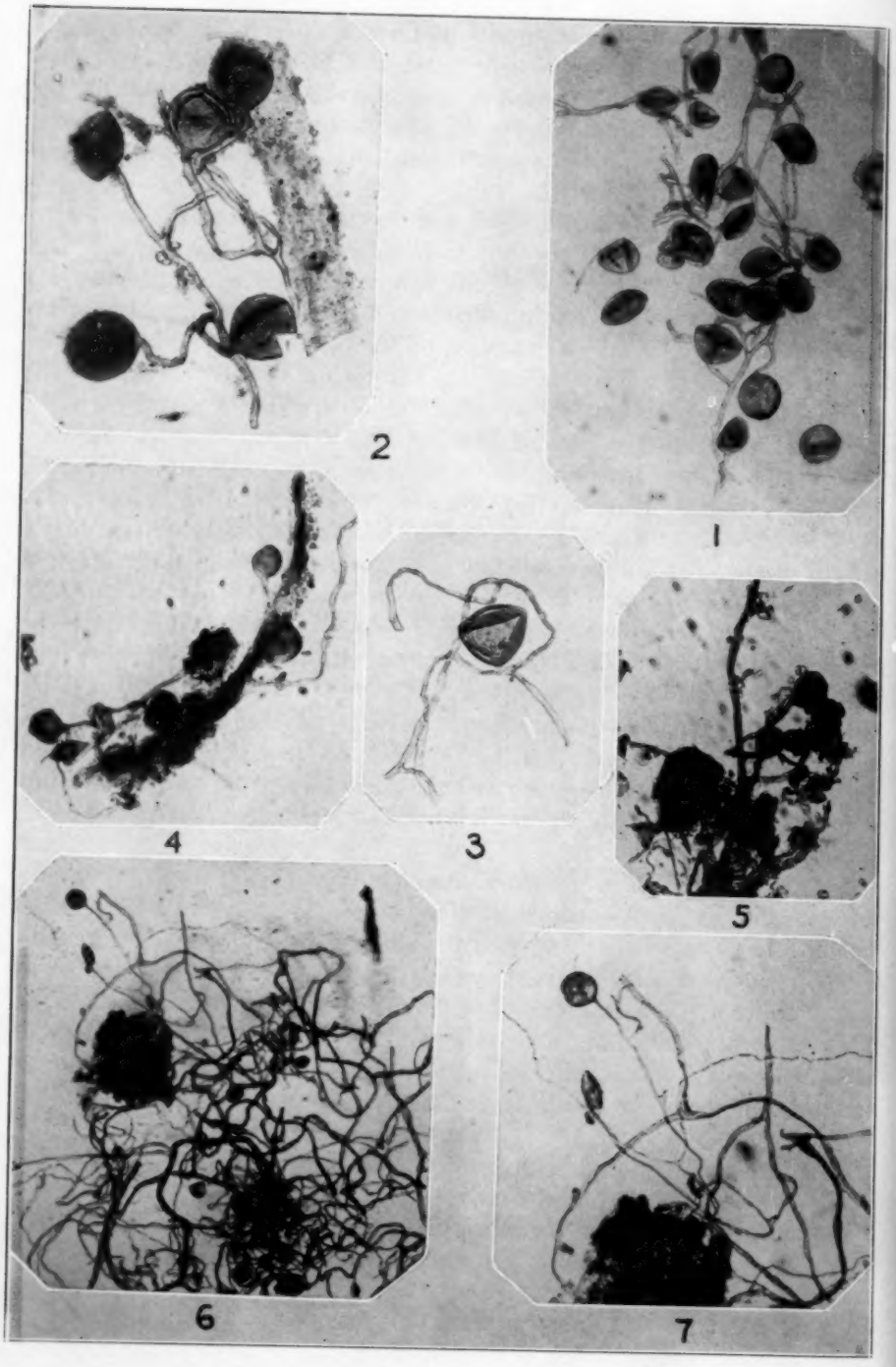
of numerous fruits of several species of *Potamogeton, Najas flexilis, Zanichellia palustris, Sparganium eurycarpum* and of several species of mat-forming sedges. Over the vegetable layer rested the boulder clay of the ground moraine of the last Wisconsin ice sheet, topped by 17 feet of glacial Lake Agassiz silt. The plant deposit therefore dates back to at least the last interglacial interval.

A year or so after the discovery of the fungus at the Bronson well site it was again found in association with a plant-bearing stratum situated 36 feet deep in Lake Agassiz silt at Moorhead, Minnesota. The vegetable layer, reported to be nearly two feet thick, was encountered in the course of excavating for a sewage disposal plant located on the banks of the Red River. The present banks of the river are considerably below the flat surface of the original Lake Agassiz bottom, yet according to Professor C. A. Ballard, of the Moorhead State Teachers' College, the silt deposit overlying the vegetable stratum showed between 1800–2000 distinct varves. Leverett and Sardeson (1932) estimate that the River Warren, the outlet of Lake Agassiz, subsided some 8000 to 9000 years ago. On the basis of this estimate it is fairly safe to assume that the vegetable layer at Moorhead, buried 36 feet deep in the lake silt, was laid down at least 11,000 years ago.

As with the Bronson well material, it was not established whether the fungus occurred only intimately associated with vegetable debris or if it was also distributed throughout the contiguous silt. The latter possibility is suggested by the fact that it was also recovered from lumps of the surrounding silt which were practically devoid of larger plant fragments. In any event, it was present in greater abundance than at the former site, but it occurred in identically the same form, i.e., in small isolated clusters of vesicles and hyphae (fig. 2).

During the past year the same organism (fig. 3) was recovered from a layer of plant remains encountered at a depth of 110 feet in a well near the village of Jackson in southwestern Minnesota. Fruits and seeds of more than a dozen species of angiosperms have been identified from this deposit, several of which are identical with those from the Bronson and Moorhead sites. The well is located on the Altamont moraine of the late Wisconsin drift and glacial deposit is of considerable depth at this place. The plant-bearing stratum can therefore hardly be of later origin than the last interglacial interval.

The fungus illustrated in Dr. Butler's text-figure 2, A–D, came from the basal one inch of amorphous peat resting upon the blue clay. Professor Lewis, who submitted the specimens from the Alberta bogs to Dr. Butler, stated that fungus material of the same character occurs in the pure clay, sometimes associated with fragments of leaves and branches of *Sphagnum*. In material of more recent peat from the same region Dr. Butler found a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closely similar fungus, but which differed from the former in having smaller vesicles that showed no tendency to form second vesicles by proliferation into the cavity of the first. The mycelium and vesicles of this form are stated to resemble in all respects the common endophyte of modern plants and in one instance the organism was found in mycorrhizal association with a root obtained at a depth of 3 dm. from the surface. It occurred throughout the peat down to a depth of 1.23 m.

The conclusions Dr. Butler reached regarding the relation of the organisms occurring in the two strata is best stated in his own words, which are as follows: "That the fungi in the glacial clay and in the overlying more recent peat are closely allied, there can be no question; except for the single fact that the older vesicles show proliferation they might be regarded as the same species."

The specimens obtained from the three Pleistocene sites in Minnesota are morphologically indistinguishable (figs. 1, 2, 3). The hyphae are somewhat tortuous, thick-walled and pale yellow in color. They vary in size but are mostly 9–11 μ in diameter. They are nonseptate, except for the rare occurrence of a cross wall in the stalk of the vesicle. The branching is subdichotomous, at an obtuse angle (figs. 2, 3), and there are occasional short diverticula and frequent unilateral projections at irregular intervals. The vesicles vary in shape from ovate to short pear-shaped and subspherical. They range in size from 75 to 103 μ in diameter and from 79 to 124 μ in length. The average of a fairly large number of measurements is 89 × 97 μ. Under medium magnification of a binocular dissecting microscope they appear dull brown in surface view, but in the sharp focus of a compound microscope the thick wall has a distinctly lemon-yellow color. Many of the vesicles were observed in open communication with the hyphae, but a few, apparently fully mature, were found occluded by a basal plug as shown in Dr. Butler's text-figure 2-A, and in some by a septum a short distance down in the stalk. The formation of a second vesicle within the first by proliferation of the stalk, in the manner described by Dr. Butler, was not seen in any of the material, although two vesicles showed a closely similar behavior. In

Explanation of figures 1–7

FIGS 1–3. *Rhizophagites butleri.* FIG. 1. Cluster of vesicles and hyphae from the Bronson well deposit, depth of 88–89 feet. × 50. FIG. 2. Smaller cluster from a layer of plant remains in Lake Agassiz silt at Moorhead, Minesota, depth of 36 feet. A portion of a decaying root is shown at the right. × 91. FIG. 3. Specimen from a well near Jackson, Minnesota, depth of 110 feet. The wall of the single vesicle is somewhat collapsed. × 91.

FIGS. 4–7. *Rhizophagites minnesotensis,* showing several clusters of hyphae and vesicles, all obtained from an early Pleistocene deposit at Springfield, Minnesota. FIG. 4. Fungus in association with a root. × 91. FIG. 5. shows two vesicles intact but much blackened. × 91. FIG. 6. shows a cluster of tangled hyphae and many vesicles, some of which are partly collapsed. × 50. FIG. 7. shows two vesicles from upper left-hand corner of figure 6, more highly magnified, also shows more clearly the variation in thickness of the hyphae and their manner of branching. × 91.

most of the older vesicles the contents become surrounded by a second wall which merges with the original wall at the neck of the stalk as shown in figures 13 and 14, or it may even extend funnel-like a short distance down into the stalk. In the latter case a thin septum may or may not be present in the stalk. This second wall ultimately becomes nearly as thick as the outer one, and the thickening obviously begins at the distal end of the vesicle and progresses towards the base (fig. 2). In a few vesicles the contents appeared as a spherical mass surrounded by an evenly thick second wall without any visible connection or contact with the outer wall (fig. 3). These differences in the internal appearance of the vesicles probably denote only different stages of their development, yet the presence of basal plugs, and of septa in some vesicles and not in others, shows that structural variations occur. The proliferation of the stalk observed by Dr. Butler is probably a more pronounced manifestation of the phenomenon.

Even though it has not been possible to duplicate in any of the Minnesota material this proliferating process, the organism otherwise agrees so completely with that from the Alberta bogs that it is fairly certain the same species of fungus is involved. The fact that it occurred widely in similar habitats, i.e., in glacial clays associated with remains of other plants, and has not hitherto been observed in material of more recent origin, indicates that it represents a species that flourished under the conditions of the Pleistocene and which probably became extinct at the end of that period.

While there is as yet no record of the certain occurrence of the species earlier than the last interglacial interval, evidence has recently come to light of the existence of the group at the beginning of the Pleistocene. This rests on the discovery two years ago of a very closely similar organism (figs. 4–7) in a piece of silt-imbedded peat that came from the line of contact between Cretaceous shale and Nebraskan till near the bottom of a preglacial gully on the property of the Ochs Brick Company at Springfield, in Brown County, Minnesota.

The hyphae of the fungus from this site are somewhat more irregular in form than the one discussed above (fig. 17), but they show the same characteristic branching, irregular projections, and color as the other. The vesicles are darker in color, less numerous, and considerably smaller (figs. 4–6), ranging from 42×46 μ to 58×61 μ. They show the same type of second wall formation within as described above (figs. 15, 16). Both hyphae and vesicles look somewhat battered as compared with those from the younger sites, but it is surprising that any structure so fragile could have endured so long in the compacted peat without complete collapse or disintegration. The peat in which the organism was found was made up largely of mosses of which the most abundant were *Camptothecium woldenii, Campylium stellatum, Swartzia montana, Scorpidium scorpioides,* and *Drepanocladus* sp. It also contained pieces of wood and other plant fragments.

In a letter to the writer Dr. Butler states that he was exhorted by Professor W. H. Lang not to give the same generic name to a recent and a fossil fungus. However, not being completely satisfied that the fungus figured in his text-figure 2 was a fossil, since it came from the amorphous peat overlying the glacial clay, he chose to assign it to a living genus under the designation of *Rhizophagus* sp. Because of the identity of the Alberta specimens with the Pleistocene material from the Minnesota sites, and furthermore the occurrence at Springfield of another species of the same genus from the early Pleistocene, there is no longer any doubt about the fossil nature of the group. Accordingly, I am describing it as a fossil genus under the name *Rhizophagites*, suggested by Dr. Butler.

Rhizophagites Rosendahl, gen. nov. Mycelium consisting of subdichotomously branched, more or less tortuous, nonseptate, thick-walled hyphae, with unilateral projections, pale yellow to light brown in color and varying in thickness from 6.5 to 20.7 μ, producing terminally ovate or short pyriform or subspherical, yellowish brown to dark brown vesicles, varying in size from 42×46 μ to 103×124 μ, with walls considerably thicker than the walls of the mature hyphae, vesicles at first in open communication with the hyphae, later becoming variously occluded by basal plugs or septa at the neck of the stalk or in the stalk or by a second wall forming around the contents, sometimes a second vesicle is formed within by proliferation from the stalk; contents made up of numerous granules, oil globules and a number of angular crystal-like bodies, vacuoles occasionally present.

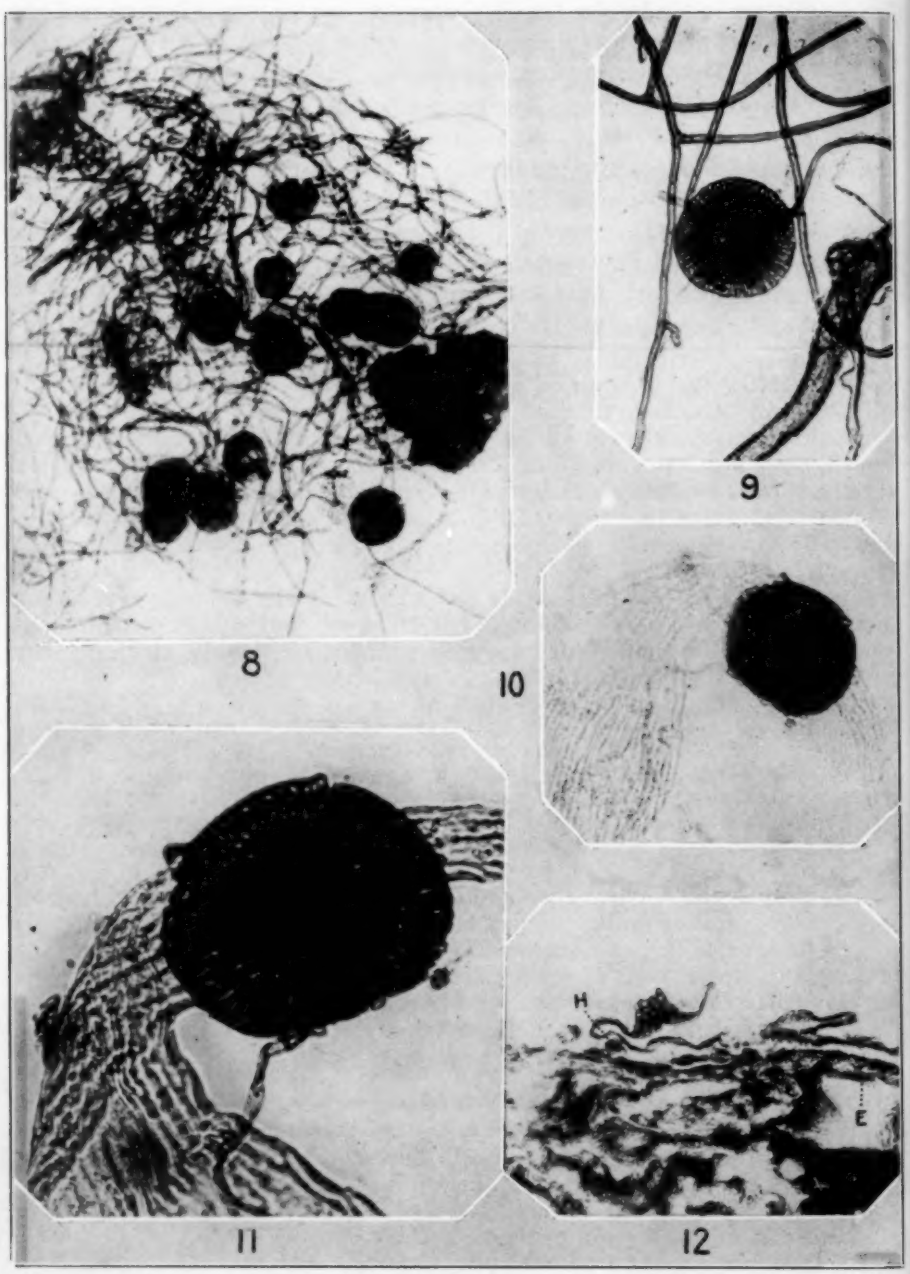
As here defined the genus consists of two species which may be distinguished as follows:

1. Vesicles light brown 75×79 μ to 103×124 μ, hyphae moderately tortuous, mostly 9–11 μ in diameter. *R. butleri.*
2. Vesicles dark brown 42×46 μ to 58×61 μ, hyphae very tortuous and irregular, 6.2–20.7 μ in diameter. *R. minnesotensis.*

Rhizophagites butleri Rosendahl, sp. nov. Hyphae moderately tortuous, with numerous unilateral projections and occasional diverticula, pale yellow in color, 9–11 μ in diameter and unform in thickness except where the branching occurs, stalks of the vesicles about the same diameter as the rest of the hyphae, vesicles chestnut-brown in color, oval to subspherical, varying in size from 75×79 μ to 103×124 μ, average size 89×98 μ, walls at the base of mature vesicles and walls of the neck of the stalks much thickened.

Rhizophagites minnesotensis Rosendahl, sp. nov. Hyphae very tortuous and with few unilateral projections, yellowish brown in color, uneven in thickness, varying from 6.2 to 20.7 μ in diameter, stalks of the vesicles thin and mostly elongated, vesicles dark brown in color, short pyriform to nearly spherical, varying in size from 42×46 μ to 58×61 μ, average size 48×53 μ, walls of mature vesicles not perceptibly thicker at the base.

The collections of the first species from the Alberta bogs and Moorhead, Minnesota, are of late Pleistocene origin, having apparently been deposited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in the silt of glacial lakes fronting the last ice sheet during its recession. The material from the deep wells at Bronson and Jackson dates back to the last interglacial interval. The collection of the second species is referable to early Pleistocene, being obtained from dense peat on the contact line between Cretaceous shale and Nebraskan till at Springfield, Minnesota.

In the same peat material from which the second species of *Rhizophagites* was obtained, well preserved fruiting bodies of another fungus were discovered. The first specimen found occurred free and consisted of a tangle of dark brown hyphae and fifteen circular perithecia (fig. 8). A second but smaller specimen was found attached to the tip of a moss plant (*Hypnum*?), but on account of the poor conditions of the temporary preparation it could not be determined whether any of the hyphae were in organic connection with the leaves and branches of the moss or were merely entangled in them. The dark brown circular fruiting bodies with a radiate cell structure and a central pore (figs. 9, 18) suggested that the organism belonged to the Microthyriaceae, and my inclination was to regard it as probably a fossil species of the genus *Microthyrium*, or in the event that the clusters of tangled hyphae belonged to the organism and represents free mycelia, then it might more properly be assigned to *Calothyrium*. On closer examination of the perithecia, however, it was discovered that they were not dimidiate but complete,[3] being provided with a well differentiated lower (fig. 19) as well as upper wall or membrane, the latter bearing a conspicuous central papilla with an apical pore (fig. 18). On account of the pressure to which they had been subjected under the load of glacial till they were very much flattened and in sectional view appear like a saucer with the central papilla rising slightly higher than the rim (figs. 12, 20). Except for the absence of asci and the dissolution of some of the delicate periclinal walls in both the upper and lower membranes the cell structure was found to be remarkably well preserved. Fungi with perithecia of this type have been segregated into the family Trichothyriaceae by Theissen (1914), and despite the fact

[3] I am indebted to Miss Louise Dosdall of the Division of Plant Pathology, University of Minnesota, for helpful suggestions in placing the fungus.

Explanation of figures 8–12

FIGS. 8–12. *Trichothyrites pleistocaenica.* FIG. 8. Cluster of perithecia with a tangle of hyphae most of which belong to a *Herpotrichia*-like form. (See text.) ×97. FIG. 9. Single perithecium more highly magnified. ×225. FIG. 10. Single perithecium on a moss leaf. Hyphae are shown faintly outlined on the surface of the leaf. ×205. FIG. 11. Same perithecium more highly magnified. ×415. FIG. 12. Cross section of spruce leaf showing two perithecia in vertical section. The one on the left is broken open along the right-hand margin. Epidermal cells of spruce leaf shown at E and fungal hyphae cross section at H. ×230.

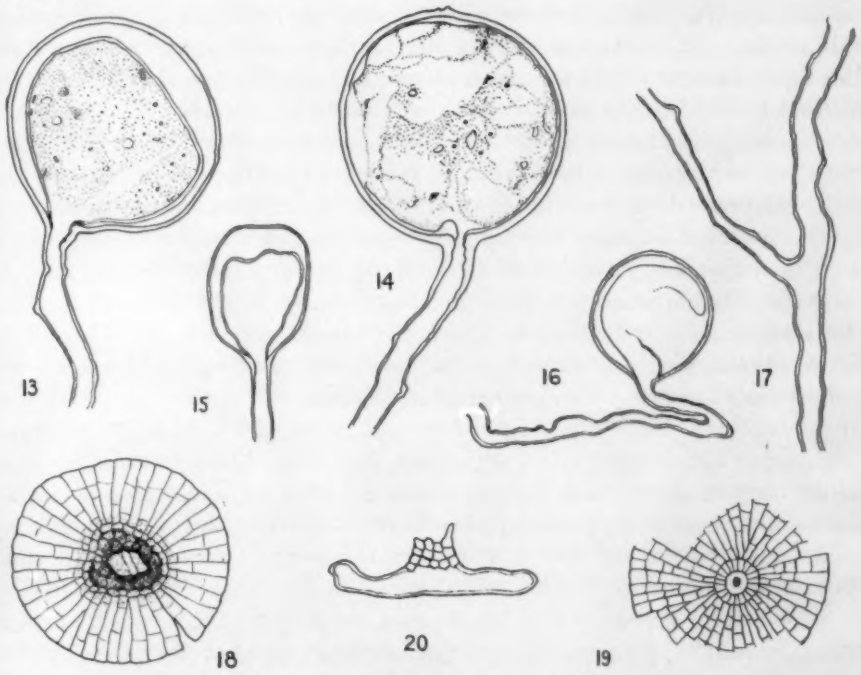
All the material for photo-micrograph figures 1–11 was prepared by mounting unstained in lactic acid and sealing the cover slips with Clarite. Figure 12 is from an unstained paraffin section. Wratten M plates and a Wratten medium yellow filter, together with a disc of daylight glass, were used in photographing.

that the living representatives of the family, so far as reported, occur almost wholly as parasites upon fungi of tropical flowering plants, the diagnostic characters set forth by Theissen would place the organism in this group. That it existed under cool or even cold conditions is clear from the fact that the peaty material from which it was recovered contained an abundance of spruce and tamarack needles, fragments of spruce wood and bark, and several species of mosses belonging mainly to the genera *Hypnum*, *Callier-gon*, *Scorpidium*, *Camptothecium*, *Swartzia*, *Ditrichum*, and *Hylocomium*. *Swartzia montana*, which is characteristic of cold and subalpine places was encountered frequently and fruits of *Potamogeton* and achenes of two or three northern species of *Carex* were also fairly common in the plant debris.

Attempts were next made to connect the fungus with a possible host and to that end all the material was carefully re-examined. The first clue obtained was the discovery of patches of dark brown fungal hyphae on many of the spruce leaves. A few of these patches were removed and mounted, and it was found that they consisted of a thin weft of interlacing hyphae with denser knots at regularly spaced intervals. Also, in a few of the preparations the above mentioned circular fruiting bodies were found more or less enmeshed in the tangle of hyphae. Some of the spruce needles were thereupon imbedded in paraffin and cross sectioned, and it was revealed that the denser knots of the investing weft were situated directly above the stomata of the leaves, which explains their orderly arrangement in parallel rows observed in the free-hand preparations. These knots are composed of densely interwoven, crinkly hyphae and some of them attain considerable size and are nearly hemispherical in shape. The habit and structure suggest a species of *Herpotrichia*, but in the absence of really diagnostic characters the identity of the organism necessarily remains in doubt.

Sections that encountered the circular fruiting bodies showed these sometimes superimposed on the thin layer of investing hyphae (fig. 12) and occasionally over the knots, but in no case could any actual connection be made out between the perithecia and the underlying or contiguous hyphae. It is fairly certain that two different fungi are involved in the picture, but what relation, if any, they bear to each other is entirely problematic. In line with the reported fungicole habit of the group it is conceivable that the trichothyriaceous organism is parasitic upon the other fungus. However, doubt is thrown upon this assumption from the subsequent discovery of the actual attachment of hyphae and circular perithecia of the same organism to the stems and leaves of mosses (fig. 10) occurring in the deposit. Both in surface view and in cross sections the hyphae were found to be closely appressed to the leaves and stems of the mosses and there can scarcely be any doubt about the fungus vegetating upon the moss plants, but whether as a parasite or as a saprophyte it is obviously impossible to say. The mycelium

is sparse (fig. 10), the hyphae are all uniform, and there is no indication of another fungus being present on the moss as in the case of the spruce needles. Connections between the mycelium and the perithecia could not be demonstrated, although in figure 11 a hypha is shown still in contact with a perithecium.



FIGS. 13, 14. Vesicles of *Rhizophagites butleri* from the Moorhead and Bronson well sites respectively. Figure 13 shows a thick second wall formed around the contents and extending into the neck of the stalk. FIGS. 15, 16. Vesicles of *Rhizophagites minnesotensis*. FIG. 17. Hypha of same, showing variation in size and sub-dichotomous branching. FIGS. 18, 20. *Trichothyrites pleistocaenica*. FIG. 18. View of upper wall or membrane of perithecium showing radiate structure and central papilla with a definite ostiole. FIG. 19. Lower membrane, incomplete but with the central portion intact, showing a very regular radiate arrangement of the cells around a larger circular central cell. FIG. 20. Perithecium sectioned vertically but the section is to one side of the middle of the papilla. Note the small, thick-walled cells of the papilla and the finger-like process of one of the border cells of the ostiole.

All figures were drawn with the aid of camera lucida to a magnification of ×660, reduced to half size in reproduction.

The perithecia from the moss and the spruce needles appear completely identical. They are the same in color, size, and every detail of structure, even to the trichome-like cells (fig. 20) that fringe the opening of the papilla, and I am convinced that they belong to the same species of fungus. The fact that the organism occurs on such widely divergent hosts as spruce leaves (or

another fungus on the spruce leaves) and at least two different species of mosses would indicate that it is a saprophyte, capable of living upon a variety of substrata in the litter of the Pleistocene bog. So far as it has been possible to ascertain, no existing fungus of this group has hitherto been reported from cool northern bogs, and the chances are that it represents an extinct species. There are several published reports of the occurrence of this general type of fungus during the Tertiary, ranging all the way from the Eocene to the Pliocene, and it is perhaps not without significance that at least three of the fossil finds were on needles of conifers. The first of the reports is by Pampaloni (1902), who described *Microthyrites dysodilis* from the Miocene of Sicily. Engelhardt and Kinkelin (1908), in their study of the upper Pliocene flora of the lower valley of the Main, reported the occurrence on *Ilex* leaves of a fungus with shield-shaped perithecia and a free mycelium which, on the interpretation of Dr. Möbius, they referred to *Asterina* (?) *ilicis* Ell. They pointed out, however, that because of the absence of spores the identification could not be considered as entirely certain. Their text-figure shows a perithecium with a slightly irregular margin and a small undifferentiated opening which suggests the absence of a true ostiole. They also found on *Buxus* leaves, in the same deposit, fruiting bodies of an Asterina-like fungus but without a free mycelium. Nathorst (1915) likewise referred doubtfully to *Asterina* a fungus obtained from cuticle preparations of leaves and twigs of *Sequoia langsdorfii* from the Tertiary of Elsmereland.

In his "Nachträge zur Tertiarflora Schlesiens" Kräusel (1920) recounted the finding of round, shield-shaped bodies, about 70 μ in diameter, in association with the leaves of *Sequoia langsdorfii*. On account of their being joined together by hyphal threads he concluded that these bodies belonged to a fungus, and he stated that they resembled in high degree the perithecia (*Gehäusen*) of the Microthyriaceae. He regarded them as being very closely similar to the form reported on *Ilex* leaves by Engelhardt and Kinkelin. It should be noted, however, that a perithecium illustrated by Kräusel has a definite ostiole, an entire margin, and is composed of fewer but relatively larger cells than the one shown in the illustration of Engelhardt and Kinkelin. According to Edwards (1922), Kräusel's material was of Miocene age.

Two years after the publication of Kräusel's paper, Edwards (1922) reported the discovery of a microthyriaceous fungus from the Eocene of Scotland. This too was obtained from conifer needles. The material ostensibly was in better shape than in the collections cited above, for it was described in much greater detail and was definitely disposed of as *Phragmothyrites eocaenica*. Edwards' photomicrographic illustration of a mature perithecium is strikingly like the figure of the fungus that Engelhardt and Kinkelin referred doubtfully to *Asterina ilicis* Ell., and the close similarity in the

structure of perithecia suggest that the two probably belonged in the same genus. The assignment to *Asterina ilicis* Ellis was unfortunate, however, since Theissen (1913) has subsequently shown that the latter belongs to the *Discomycetes*.

In all of these reports identification was apparently based only upon the surface view of the fruiting bodies, but since it is next to impossible to determine from the surface aspect alone whether the perithecia (ascomata) are dimidiate or complete, it is not certain that they all belong to the Microthyriaceae. Kräusel's illustration of one of the circular bodies found in his fossil material bears a very close resemblance to the perithecia of the Pleistocene fungus under discussion, and it is not improbable that the organisms involved are congeneric. Whether or not this is the case the Minnesota material can not be referred to any of the previously named fossils, and I am venturing to describe it as a fossil genus belonging to the Trichothyriaceae. The existing genera of the family are segregated upon spore characters, and since spores are entirely lacking in all the material seen it can hardly be treated otherwise than a form genus for which the name *Trichothyrites* seems the most appropriate.

Trichothyrites pleistocaenica Rosendahl, gen. et spec. nov. Mycelium[4] consisting of yellowish or brownish, branching, septate hyphae with occasional anastamoses, 5–6.7 μ in diameter, individual cells (20) 28–33 (41) μ long, perithecia dark brown to nearly black, circular or nearly so, disk-shaped, or because of slightly upturned margin shallow saucer-shaped, 70–95 μ in diameter, complete, with upper and lower membranes composed of radially arranged cells, upper membrane with a central papilla having a distinct pore or ostiole, marginal cells of membrane 4–5 μ wide, 6–8 μ long, cells of papilla more nearly quadrangular and thick walled, several of the marginal cells of pore prolonged into finger-like processes, cells of lower membrane all thin walled and radiating from a circular central cell, asci and spores lacking.

Found on leaves of Black Spruce in association with an unknown fungus and on two species of hypnaceous mosses, in an early Pleistocene deposit at Springfield, Minnesota.

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[4] Description of mycelium drawn from fungus on moss plants. On the spruce leaf materials it is obscured by the tangled hyphae of the other fungus present.

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ASAHINA'S MICROCHEMICAL STUDIES ON THE CLADONIAE[1]

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Asahina's microchemical studies on *Cladonia* (and other critical lichen-genera) have made it possible for the taxonomist to demonstrate the presence of numerous lichen-acids and allied substances without recourse to the complicated methods employed by the organic chemist. The results of these studies are given in several special articles and in two series of contributions: (1) *Lichenologische Notizen* (2, 8, 13),[2] the first number of which appeared in 1933; and (2) *Mikrochemischer Nachweis der Flechtenstoffe* (1, 3, 4, 5, 6, 7, 9, 10, 12, 15), the first number of which appeared in 1936. Of the first series fifteen numbers have been published; of the second, eleven. Of the special articles the following have a direct bearing on North American *Cladoniae*: *Ueber den Chemismus der Flechten der Cocciferae* (11); *Japanische Arten der Cocciferae* (13); *Cladonia verticillata Hoffm. und Cladonia Calycantha (Del.) Nyl. aus Japan* (17); and *Cladonia chlorophaea und verwandte Arten* (18).

Asahina's work up to 1938 has been reviewed by Sandstede in his supplement to Vainio's monograph on the *Cladoniae* (19), but there are few other references to it in the recent literature on lichens. It seems advisable, therefore, to bring to the attention of North American students the more important features of his methods and to summarize the results which he has already obtained. In doing this no attempt will be made to discuss the chemical structure of the various substances involved. Those interested in this aspect of the subject may consult Zopf's comprehensive study on lichen-substances (20) or Asahina's recent classification of these substances from a chemical standpoint (14).

In order to demonstrate the presence of a definite lichen-substance in a specimen Asahina's methods A and B (1, pp. 519, 520) are applicable in most instances. These methods involve two steps or processes. In the first step a portion of the lichen to be tested is placed on a slide and extracted by means of a volatile solvent, such as acetone or chloroform, applied drop by drop; and care must be taken to avoid an excess of the solvent, otherwise the solution will spread over too large an area. In the case of the *Cladoniae* either the primary squamules or the podetia may be investigated. If the

[1] Contribution from the Osborn Botanical Labortory.

[2] The numbers refer to the bibliography at the end of this paper. The only titles listed are those which refer, directly or indirectly, to species of *Cladonia* known to occur in North America.

podetia are small it may be necessary to use two or more, but if they are large a single podetium or even a portion of a podetium will be sufficient. Upon the evaporation of the solvent and the removal of the lichen-sample, a dry extract is left on the slide, usually in the form of a whitish or yellowish coating or crust, in which as a rule little of a definite nature can be distinguished under the microscope.

The material is now ready for the second step. This involves the use of a second solvent, from which the substance or some compound of the substance will crystallize out in a recognizable form. A drop or two of the solvent should be placed on the dry extract and a cover-glass applied. Then, in order to facilitate the solution it is usually necessary to apply a gentle heat, and Asahina recommends for this purpose a microflame, either of a gas burner or of an alcohol lamp. If care is taken, however, a lighted match may be sufficient. As the preparation cools the crystals gradually make their appearance. This may take place after a few minutes but may require an hour or more in the case of certain subtances. The solvents used in carrying out the second step include the following, designated by the abbreviations which Asahina employs (1, p. 519; 3, p. 860; 4, p. 529) :—

G.A.W., one part each of glycerine, alcohol, and water;
G.E., one part glycerine and three parts glacial acetic acid (Eisessig);
G.W.Py., one part glycerine, three parts water, and one part pyridine;
G.A.Q., two parts glycerine, two parts alcohol, and one part quinoline;
G.A.An., two parts glycerine, two parts alcohol, and one part aniline; and
G.A.o-T., two parts glycerine, two parts alcohol, and one part ortho-toluidine.

These various solutions, together with the acetone and the chloroform, should be kept in small bottles, the necks of which are closed with rubber medicine-droppers. By this means the reagents may be applied drop by drop, and the danger of adding an excess can be avoided.

Although most of the substances to be tested for are readily soluble in cold acetone, a few require hot or even boiling acetone before they can be brought into solution. Since acetone is inflammable and boils at a relatively low temperature (about 56° C) the heat must be carefully applied. Asahina (4, p. 530) recommends for this purpose a glass tube about 10 cm. long and 8 mm. in diameter. The lower end of this tube is gradually narrowed to a diameter of about 2 mm., and the narrowed portion is curved back in such a way that it lies parallel with the main tube, except at the free end, which is curved outward. This apparatus thus resembles, as Asahina points out, a miniature burette of the Gay Lussac type (see 4, *f. 33–35*). The lichens or lichen-fragments to be extracted are pressed down into the tube, covered with acetone, and heated over a microflame. In doing this the tube should be held obliquely and care should be taken not to expel the contents by applying the heat too abruptly. After the acetone has boiled gently for a minute or

two the contents of the tube should be poured out through the narrow end upon a slide and allowed to evaporate to complete dryness. The extract thus obtained, which tends to spread out over a considerable surface, should be scraped together by means of a scalpel before proceeding to the second step in Asahina's method of crystallization.

The crystals of certain lichen-substances are large enough to be recognized under the low power of the microscope, but the high power is necessary in most cases to bring out their distinctive features. There are a few substances, in fact, in which the crystals are so very minute that they can be easily overlooked. If the presence of a given substance is suspected in a lichen and if negative results are obtained at the first trial, a second or even a third extraction may be advisable before deciding that the substance is absent. It must be kept in mind also that the presence of certain substances may make the demonstration of other substances by microchemical methods difficult if not impossible.

At the present time 19 lichen-acids and related substances, which can be demonstrated by Asahina's methods, have been reported in the *Cladoniae*. These substances, which are arranged below in alphabetical sequence, will now be considered with respect to the following data: (1) the distribution of the substance among the various North American species of the genus; (2) the reagents to be used in its demonstration; and (3) the characteristic features of the crystals obtained. In this resumé many of the reactions described by Asahina are omitted and the attempt is made to emphasize those of the most practical importance from the standpoint of the taxonomist. Additions to the list will doubtless be made in the near future.

1. Atronorine. This substance is widely distributed among the lichens and has been reported in the following species of *Cladonia: C. caespiticia* (Pers.) Floerke, *C. cariosa* (Ach.) Spreng., *C. Evansii* des Abbayes, *C. furcata* (Huds.) Schrad., *C. gracilis* (L.) Willd. var. *elongata* (Jacq.) Floerke, *C. major* (Hag.) Sandst., *C. papillaria* (Ehrh.) Hoffm., *C. rangiferina* (L.) Web., *C. rangiformis* Hoffm., and *C. symphycarpia* Floerke. It will be noticed that none of these species are red-fruited. Atronorine is negative with P[3] but gives a pale lemon color with K, very different from the deep yellow produced by thamnolic acid. In species with persistent primary squamules the reaction with K shows particularly well if the reagent is applied to the chalky lower surface.

Perhaps the best way to demonstrate atronorine microchemically is to extract the lichen-sample with acetone and to apply the G.A.o–T. solution to the dry residue. If the preparation, after careful heating, is examined under the microscope slender yellow crystals are soon seen to make their

[3] The abbreviation "P" stands for paraphenylenediamine, the abbreviation "K," for potassium hydroxide.

appearance (see Asahina, 4, p. 535, *f. 43*, *pl. 2*, *f. 6*). These are in the form of very narrow plates with parallel sides and may present the appearance of needles if seen on edge. The crystals, if occurring singly, are usually variously curved or contorted and show more or less irregular branching at their ends. They are most characteristic, however, when they form radiating clusters. Under these circumstances the crystals tend to branch repeatedly at their free ends and to give rise to intricately interwoven clusters of thread-like elements. Another excellent method of demonstrating atronorine is by treating the dry acetone extract with a saturated solution of barium hydroxide (3, p. 961, *f. 15*). After heating gently the barium salt crystallizes out in the form of dense spherical clusters of delicate yellow lamellae, which look like fine needles if examined casually.

2. BAEOMYCIC ACID. This acid, which was first isolated from *Baeomyces roseus* Pers., has been found by Asahina in a single species of *Cladonia*, *C. strepsilis* (Ach.) Vainio (9, p. 652), and is responsible for the yellow color which this species shows upon treatment with P. The pure acid yields characteristic crystals with various reagents and the same results can be obtained with the acetone extract of the *Baeomyces*. The G.A.Q. solution, for example, brings about the formation of minute, very pale yellow, thin rhomboidal plates, occurring singly or in irregular groups. In some cases, as shown by one of Asahina's figures (9, *f. 84*) the ends of the rhombi are shortly truncate, but the majority are regularly formed (9, *pl. 5*, *f. 3*). The presence of strepsiline, unfortunately, which accompanies the baeomycic acid in *C. strepsilis*, interferes with its crystallization, and Asahina gives no method by means of which the strepsiline can be first separated.

3. BARBATIC ACID. Barbatic acid was first extracted in 1880 from material which had been determined as *Usnea barbata* (L.) Wigg., but the identity of this material is still in doubt. At a later date Zopf (20, p. 238) reported the occurrence of the acid in several other lichens, including *U. ceratina* Ach. and *Alectoria ochroleuca* (Ehrh.) Nyl. Asahina (3, p. 870) has shown, however, that the acid present in these two species is not barbatic acid but diffractic acid, a substance first isolated from the Japanese *U. diffrata* Vainio. The distribution of barbatic acid, therefore, among the species of *Usnea* and *Alectoria* awaits further investigation. In the genus *Cladonia* barbatic acid has been reported as a definite component of the following species (see 11, p. 24): *C. amaurocraea* (Floerke) Schaer., *C. bacillaris* (Ach.) Nyl., *C. coccifera* (L.) Willd., *C. cristatella* Tuck., *C. didyma* (Fée) Vainio, *C. Floerkeana* (Fr.) Floerke, and *C. macilenta* Hoffm. It will be noted that all of these species, with the exception of *C. amaurocraea*, are members of the section *Cocciferae*. Barbatic acid, as shown by Asahina (3, p. 868, and 5, p. 856), is indistinguishable from the coccelic acid of Hesse and from a substance distinguished by Zopf (21, p. 55) under the name

cenomycine. Both of these substances were listed by Zopf under most of the species enumerated above.

For the demonstration of barbatic acid, which is not difficult, either the G.E. solution (3, p. 868) or the G.W.Py. solution (5, p. 856) may be applied to the dried acetone extract. If the G.E. solution is used the acid, after gentle heating, will appear in the form of short, refractive, colorless rhombic prisms or rectangular lamellae (3, *f. 27*), associated in some cases with boat-shaped prismatic structures having pointed ends (5, *pl. 5, f. 3*). The crystals obtained by means of the G.W.Py. solution, likewise after careful heating, are perhaps even more satisfactory. These crystals are in the form of colorless, narrow, four-sided lamellae (5, *f. 44, pl. 5, f. 4*). In their most typical condition they are oblique at the ends and rhombic in outline, but the ends may be square or angled, in the latter case showing a combination of the oblique and square conditions. The most characteristic crystals, however, are united in pairs, and it is usual for one or both members of the pair to project beyond the line of junction. Twin crystals of this type can readily be found in most preparations and are particularly striking if the line of junction is relatively short and if the crystals project in opposite directions.

4. Bellidiflorine. Zopf, who isolated bellidiflorine from the northern *Cladonia bellidiflora* (Ach.) Schaer. in 1907 (20, p. 332), was unable to find it in any other member of the genus (21, p. 108). Asahina, however, demonstrated its presence in the following North American species, all belonging to the section *Cocciferae* (11, p. 24; 14, p. 603): *C. digitata* (L.) Hoffm., *C. deformis* Hoffm., *C. endoxantha* Vainio, *C. gonecha* (Ach.) Asahina (a recent segregate from *C. deformis*), *C. incrassata*,[4] *C. leporina* Fr. (13, p. 604), *C. macilenta*, *C. pleurota* (Floerke) Schaer., and *C. polydactyla* Floerke. In a few of the species bellidiflorine occurs only as an "accessory" substance, i.e., it is not invariably present.

Bellidiflorine dissolves with difficulty in cold acetone but readily passes into solution if the solvent is boiled. The method described on page 140 should therefore be employed. If the dried extract thus prepared is treated with the G.A.An. solution and gently heated (10, p. 771), the very characteristic crystals of the aniline salt are obtained. These crystals do not make their appearance immediately but only after a considerable time. They are in the form of minute, six-sided lamellae (10, *f. 99, pl. 6, f. 4*), which show a brownish yellow or brownish green color. In most cases the sides of the hexagons are equal, but some irregularity is to be expected.

5. Cryptochlorophaeic acid. According to our present knowledge this acid is confined to *Cladonia cryptochlorophaea* Asahina, a recent segregate from *C. chlorophaea* (Floerke) Spreng. (see 18, p. 710).

[4] The authorities for specific names are given only when the species are first mentioned.

The dry extract obtained by treating podetia of *C. cryptochlorophaea* with acetone is abundant, somewhat opaque, and more or less tinged with yellowish or brownish in transmitted light. It consists in most cases of several concentric bands and shows at the periphery a series of rounded projections. If the G.A.W. solution and gentle heat are applied to the extract the preparation forms upon cooling the characteristic colorless crystals of cryptochlorophaeic acid, which are in the form of long and exceedingly fine needles. When these occur singly they tend to be more or less curved, and both extremities become repeatedly subdivided. It is more usual, however, for the crystals to be arranged in dense, radiate clusters around a central point (18, *pl. 1, f. 1*). Under these circumstances they remain undivided at their attached ends but branch repeatedly at their free ends, the branches becoming finer and finer. In this way circular aggregates of crystals may be formed. These bear a certain resemblance to the crystal-aggregates which atronorine forms in the G.A.o-T. solution, but the latter are distinctly yellow, their larger elements are in the form of narrow bands, and their finer branches tend to be more intricately interwoven and distorted.

6. DIDYMIC ACID. Asahina announced the discovery of didymic acid in 1939 (11, p. 32) and demonstrated its presence in the following North American species of *Cladonia*, all belonging to the section *Cocciferae: C. cristatella, C. didyma, C. leporina,* and *C. vulcanica* Zolling. A short time later he added *C. incrassata* to the list (12, p. 467) and showed that his didymic acid was identical with the incrassatic acid of Zopf. The species in which didymic acid has so far been found are all members of the section *Cocciferae.*

The acid is readily soluble in acetone, and distinctive crystals are obtained if the dried extract is treated with the G.E. solution and gently heated. It is advisable to use for this purpose a sufficiently large lichen-sample. The crystals, which gradually appear after cooling, are colorless and in the form of long and narrow rhombic lamellae with oblique ends (12, *f. 100*). Many of the crystals, if seen on edge, present the appearance of needles, slightly thicker in the middle and tapering to long points. The crystals occur singly, in irregular groups, or in radiate or penicillate clusters (12, *pl. 1, f. 1*). Excellent results may be obtained also if the extract is similarly treated with the G.A.W. solution. The crystals resulting from this treatment are similar to those just described (12, *pl. 1, f. 2*) but, in the writer's experience, even more slender and needle-like. Radiate clusters of these crystals are frequent, although irregular groups and single crystals also occur abundantly. Asahina emphasizes the fact that the crystals may be curved or hook-like at the ends. He figures also abnormal S-shaped crystals with the ends variously subdivided (12, *f. 101*).

7. FUMARPROTOCETRARIC ACID. Zopf (20, p. 173) notes the occurrence of this bitter substance in several genera of the lichens, but it seems to be espe-

cially widely distributed in the genus *Cladonia*. Even here, however, it is restricted to the subgenus *Cladina* and the subsections *Chasmariae* and *Clausae*. It is therefore lacking in all red-fruited species and in the subsection *Unciales*. Fumarprotocetraric acid may be regarded as the characteristic acid, or as one of the characteristic acids, in the following North American species: *C. borbonica* (Del.) Nyl., *C. caespiticia*, *C. calycantha* Del. (16, p. 469), *C. chlorophaea*, *C. clavulifera* Vainio, *C. coniocraea* (Floerke) Spreng., *C. conista* (Ach.) Robbins, *C. cornutoradiata* (Coem.) Sandst., *C. fimbriata* (L.) Fr., *C. furcata* (Huds.) Schrad., *C. gracilis*, *C. major*, *C. mateocyatha* Robbins, *C. mitrula* Tuck., *C. multiformis* Merrill, *C. pityrea* (Floerke) Fr., *C. pyxidata* (L.) Hoffm., *C. rangiferina*, *C. scabriuscula* (Del.) Leight., *C. sylvatica* (L.) Hoffm., *C. tenuis* (Floerke) Harm., and *C. verticillata* (Hoffm.) Schaer. It occurs also, as an accessory substance, in *C. cryptochlorophaea*, *C. Grayi* Merrill, *C. merochlorophaea* Asahina, and *C. nemoxyna* (Ach.) Nyl.

For the demonstration of fumarprotocetraric acid the negative reaction with K and the distinctly red color produced by P are usually sufficient. If, for any reason, doubt should arise Asahina's microchemical methods may be employed. He recommends, in the case of *C. furcata*, for example (9, p. 659), the extraction by acetone diluted with 15 per cent of water. The material should be boiled in this mixture and the dried extract should be treated with the G.A.An. solution. After careful heating and subsequent cooling the aniline compound will crystallize out in the form of long and fine yellow needles, variable in length and arranged in radiating cluster. These as a rule do not form complete circles but show the needles distributed in two or more sectorial groups.

8. Grayanic acid. This acid represents the characteristic constituent of *C. Grayi*, as now defined by Asahina (see 18, p. 713), and occurs also in *C. borbonica* (18, p. 717). When Asahina first extracted grayanic acid he considered it identical with the chlorophaeic acid of Zopf (12, p. 468) but apparently now regards the latter as an impure substance.

If a podetium of *C. Grayi* is treated with acetone the dried extract already shows definite, needle-like, colorless crystals of grayanic acid. At the periphery of the preparation these usually occur in spreading or radiating groups, but in many cases they are free from one another and irregularly scattered (12, *pl. 1, f. 3*). In cases of doubt the application of the G.A.W. solution, followed by gentle heating, will bring about the appearance of longer and exceedingly fine needles, forming definite radiate clusters or irregular groups (12, *pl. 1, f. 5*).

9. Homosekikaic acid. Asahina first produced this acid synthetically and then demonstrated its presence in Japanese material of *C. pityrea* (7, p. 249). It is apparently an accessory substance in this species, since it has

not yet been found in European or North American specimens. In *C. nemoxyna*, however, homosekikaic acid is the characteristic substance present and is identical, as Asahina soon showed, with the nemoxynic acid of Zopf (8, p. 251).

In order to prove that homosekikaic acid is present Asahina's directions (7, p. 250) should be carefully followed. The dried acetone extract, as he points out, presents a varnish-like appearance, and he directs that this extract should be scraped together into a compact mass. To this a coverglass smeared with the G.A.o-T. solution should be directly applied. If the preparation is then studied, without heating, under the microscope, one or more yellowish oily masses will soon be seen to make their appearance and in these the crystals of the ortho-toluidine salt will quickly be visible. These are in the form of exceedingly thin hexagonal plates, which occur singly or in lamellate clusters (7, *pl. 4, f. 6*). The sides of an individual plate are, in most cases, unequal in length.

10. MEROCHLOROPHAEIC ACID. At the present time this acid is known only in *C. merochlorophaea*, a recent segregate from *C. chlorophaea* (18, p. 710). A sufficient amount of extract for testing can usually be obtained if a single podetium of *C. merochlorophaea* is treated with acetone, but two or more may be necessary if the podetia are unusually small. The extract presents a varnish-like appearance, and Asahina (18, p. 712) recommends further treatment with the G.E. solution, followed by gentle heating and subsequent cooling. The crystals which now appear usually radiate out irregularly from a central area and are in the form of thin colorless lamellae, with parallel sides and oblique ends, the acute angles of which measure about 50°. The angles of course appear less if the lamellae do not lie flat, and the crystals may look like needles if seen on edge.

11. NORSTICTIC ACID. This name was given by Asahina and Yanagita to a substance obtained from *Lobaria pulmonaria* (L.) Hoffm. (9, p. 655). It has since been found in *Parmelia acetabulum* (Neck.) Duby, in *Usnea japonica* Vainio, and in *Cladonia subcariosa* Nyl. The acid is responsible for the deep red color which the *Cladonia* shows upon the application of K.

Norstictic acid is readily soluble in acetone, and perhaps the easiest way to demonstrate its presence microchemically is to treat the dried extract with a solution of potassium carbonate in the presence of K. Asahina recommends for this purpose a 10 per cent solution of K_2CO_3 plus a 5 per cent solution of KOH. The potassium salt will then appear, especially after heating, in the form of short straight needles, yellowish red to red in color. These occur either separately or variously grouped and may form radiate clusters. Another solution which gives excellent results is the G.A.o-T. solution. If this is added to the dry extract pale yellow crystals will appear, especially after heating, in the form of exceedingly thin, four angled lamellae (9, *f. 90*), occurring singly or in irregular groups.

12. PERLATOLIC ACID. This substance, first extracted by Asahina and Fujikawa from *Parmelia cetrarioides* Del. var. *typica* DR. (6, p. 40), has recently been demonstrated also in two North American species of *Cladonia*, *C. Evansii* and *C. impexa* Harm. (15, p. 186, 189). The acid can be readily detected by treating the dry acetone extract with the G.A.Q. solution, followed by gentle heating. Upon cooling the characteristic crystals will appear, in some cases only after several hours, in the form of dense radiating clusters of colorless, exceedingly fine straight needles (15, *f. 3*). As a rule only one or a very few such clusters are present in a single preparation.

13. PSOROMIC ACID. Although widely distributed among lichens in general (20, p. 198) psoromic acid has been reported from very few species of *Cladonia*. At the present time, in fact, *C. alpicola* (Flot.) Vainio and *C. alpestris* (L.) Rabenh. f. *aberrans* des Abbayes are the only North American forms that can be definitely cited from the literature (21, p. 87; 2, p. 804; 9, p. 656; 15, p. 190).

Lichens containing psoromic acid are negative or nearly so with K but give a deep yellow color with P. Extraction with acetone takes place readily, and if the dried residue is treated with the G.E. solution and gently heated the psoromic acid will crystallize out upon cooling in the form of penicillate or radiate clusters of fine colorless needles (9, *f. 52*). As Asahina points out these clusters may be large enough to be visible with the naked eye.

14. SQUAMATIC ACID. This substance is apparently restricted to the *Cladoniae*. It was first extracted from *C. squamosa* (Scop.) Hoffm. but is now known also in the following additional North American species (21, p. 106; 11, p. 24): *C. bellidiflora*, *C. caespiticia*, *C. cenotea* (Ach.) Schaer., *C. crispata* (Ach.) Flot., *C. delicata* (Ehrh.) Floerke, *C. glauca* Floerke, *C. gonecha*, *C. incrassata*, *C. strepsilis*. It may be noted that some of these species are red-fruited and others brown-fruited.

Since squamatic acid is only slightly soluble in cold acetone but more readily so in hot acetone, Asahina's burette-like tube is advisable for its extraction. If the residue thus obtained is treated with the G.E. solution and gently heated, the acid will crystallize out upon cooling in the form of minute, colorless, rhombic plates or truncated, rhombic, double pyramids (6, *f. 58*). In many cases small groups of crystals coalesce in various ways and thus form irregular compound crystals. The red-fruited species listed above contain usnic acid as well as squamatic acid, and Asahina recommends in such cases the use of the G.A.An. solution. In this solution the aniline salt of squamatic acid appears in the form of colorless rhombic prisms (10, *f. 99*).

15. STICTIC ACID. The extraction of stictic acid from *Lobaria pulmonaria* (*Sticta pulmonaria* Ach.) was made nearly a hundred years ago (20, p. 204), and the substance has since been found in the genera *Parmelia*, *Ramalina*, and *Stereocaulon* (7, p. 655). Although there are no reports in the literature

of its occurrence in the genus *Cladonia*, Asahina (in a recent letter) notes its presence in one of the North American species. It is accompanied by norstictic acid, and he has already pointed out that these two acids are not infrequently produced by the same species (7, p. 655). Since Asahina has not yet published his observations on the *Cladonia* in question no further details can be given here.

The demonstration of stictic acid follows a familiar pattern. The lichen-sample is first extracted with acetone, and the residue left after evaporation is treated with the G.A.o-T. solution and carefully heated. Upon cooling characteristic crystals make their appearance. They are in the form of exceedingly thin, hexagonal lamellae, the sides of which in most cases are equal (7, *f. 89*). According to Asahina the crystals are yellow, but the color may be so pale that they appear colorless. The crystals occur singly or in indefinite overlapping groups, and the surface shows a series of irregular lines or other markings.

16. STREPSILINE. This substance, according to our present knowledge, is confined to *C. strepsilis*. When the squamules or podetia of this species are treated with chloride of lime a more or less distinct green or bluish green color is produced, and students of the genus have regarded this color as a proof that strepsiline was present. Asahina's microchemical methods, however, give more trustworthy results, since the color-reaction is not always clear. He directs (12, p. 469) that the specimen to be tested should be extracted with acetone and that the residue obtained after drying should be treated with the G.E. solution and carefully heated. Upon cooling the crystals of the strepsiline gradually make their appearance. These crystals are colorless and minute and in some cases very scantily produced. They are therefore easily overlooked and it may be necessary to make two or more extractions before a successful demonstration is obtained. The crystals (20, *f. 58;* 12, *pl. 1, f. 6*) are in the form of rhombic plates, which may, if sufficiently thick, show a pale brownish tint. The acute angles of the rhombic faces measure 68°, and the surface is usually marked with a series of fine parallel lines. The crystals occur singly or unite to form compound crystals of various forms. The most characteristic of these appear split at one or both ends, with the free portions diverging from each other.

17. THAMNOLIC ACID. This acid derives its names from *Thamnolia vermicularis* (Sw.) Ach., in which it was first found. It is now known to occur also in several other lichen-genera and in the following North American species of *Cladonia* (9, p. 651; 10, p. 768; 11, p. 24): *C. delicata, C. digitata, C. endoxantha, C. macilenta, C. polydactyla, C. Ravenelii* Tuck., *C. santensis* Tuck. (*C. persquamulosa* Merrill), and *C. subsquamosa* Nyl. The red-fruited species in this list belong to the subsection *Subglaucescentes;* the brown-fruited, to the subsection *Chasmariae*. Several other species, not yet recorded in the literature, will probably be added to the list in the near future.

The presence of thamnolic acid is shown by the bright yellow color produced by K, together with the deep orange or red color produced by P. To demonstrate the acid microchemically extraction with hot acetone is advisable, since cold acetone is almost inactive as a solvent. For further treatment the G.A.An. solution (9, p. 651) is especially to be recommended. This should be added drop by drop to the dry residue and careful heat applied. The preparation will then assume a deep yellow color, accompanied by the production of bubbles of CO_2, and characteristic crystals will make their appearance upon cooling. These are in the form of fine yellow needles, grouped together in dense radiate or fasciculate clusters (9, *pl. 5, f. 1*).

18. Usnic acid. As the name implies usnic acid was first demonstrated in the genus *Usnea*. Now, however, it is recognized as one of the most widely distributed of the lichen-acids, and Zopf (20, p. 102) notes its occurrence in nine distinct families. In the genus *Cladonia* the acid represents the characteristic substance or one of the characteristic substances in the following North American species (21, p. 106; 11, p. 24): *C. alpestris, C. impexa, C. mitis* Sandst., *C. sylvatica,* and *C. tenuis,* representing the subgenus *Cladina; C. bellidiflora, C. coccifera, C. cristatella, C. deformis, C. gonecha, C. incrassata, C. leporina,* and *C. pleurota,* representing the subsection *Stramineoflavidae* of the section *Cocciferae; C. amaurocraea, C. Boryi* Tuck., *C. caroliniana* (Schwein.) Tuck., and *C. uncialis* (L.) Web., representing the subsection *Unciales; C. foliacea* (Huds.) Willd.[5] and *C. cyanipes* (Sommerf.) Vainio, representing the groups *Foliosae* and *Ochroleucae,* respectively, of the subsection *Clausae.* It occurs also, as an accessory substance, in *C. bacillaris* and *C. Floerkeana,* representatives of the subsection *Subglaucescentes* of the section *Cocciferae* (13, p. 603). It will be seen that the foregoing list includes no representatives of the large subsection *Chasmariae* or of the large groups *Podostelides* and *Thallostelides* of the subsection *Clausae.*

Lichens containing usnic acid tend to show a more or less marked yellowish tinge, although this is by no means invariably the case. They are negative with both K and P (unless certain other acids are present also), but turn distinctly yellow with chloride of lime, especially in the presence of K. The extraction of the acid for microchemical study can be made with either acetone or chloroform, but the latter is perhaps preferable because it leaves certain other lichen-substances undissolved. The extract obtained from either solvent is pale yellow and crystalline in appearance. Further treatment, however, is necessary in order to produce satisfactory crystals of the acid and for this purpose the G.E. solution yields excellent results (3, p. 863). After careful heating and subsequent cooling these crystals gradually make their appearance and can readily be seen under the low power. They are in

[5] The occurrence of the true *C. foliacea* in North America is open to question, but a closely allied species is not infrequent.

the form of yellow needles or narrow lamellae with parallel sides, the ends of which are obliquely cut off or taper to long points. The crystals occur singly, in indefinite groups, or in loose radiating clusters (3, *f. 17*).

19. ZEORINE. Zeorine was first extracted from some species of *Zeora*, which is regarded at the present time as a synonym of *Lecanora*. It is now known also in various other genera of the Lecanoraceae, in two genera of the Physciaceae (20, p. 53), and in two red-fruited species of *Cladonia*, *C. deformis* and *C. pleurota*. If a podetium of one of these species is extracted with acetone, a residue is left which yields characteristic crystals upon further treatment with the G.A.An. solution (10, p. 770). As in other cases heat should be carefully applied to the preparation, which should then be allowed to cool. The crystals (10, *f. 97*) are minute, colorless, and highly refractive. The most typical are in the form of truncated, hexagonal, double pyramids. If such a crystal is viewed from above it shows a regular hexagonal outline, and a second hexagon can be distinguished at a higher level by careful focusing. Other crystals are in the form of complete, broad or narrow, hexagonal pyramids, and the latter not infrequently show a broader equatorial band. In some cases the sharply angled crystals are accompanied by irregular crystals in which the angles are rounded or otherwise indistinct.

Microchemical methods for the detection of certain lichen-substances found in the *Cladoniae* have not yet been described and may not be applicable. These include the following: cervicornic acid, found in the brown apothecia of certain forms of *C. verticillata* (21, p. 84); destrictic acid, found in the apothecia and spermagonia of the European *C. destricta* Nyl. (20, p. 331); fimbriatic acid, found in *C. fimbriata* and *C. major* (20, p. 107); rangiformic acid, found in *C. mitis* (19, p. 91) and *C. rangiformis* (21, p. 106); and rhodoclonic acid, found in the apothecia of the red-fruited species (21, p. 55).

Asahina's microchemical investigations afford a new method of attack for the taxonomist by enabling him to determine many chemical features of lichens with comparative ease. They do not settle, however, the controversy regarding the significance of chemical differences from a taxonomic standpoint. Some writers regard such differences, if constant, as important as morphological differences; others assign them a secondary value and accept them only when supported by morphological differences; still others consider them of no taxonomic significance whatever. Which of these views will ultimately prevail must be left for the future to decide.

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OBSERVATIONS ON THE CULTURE OF HEMITRICHIA VESPARIUM, WITH SPECIAL REFERENCE TO ITS BLACK PLASMODIAL COLOR

RUTH N. NAUSS

The distinguishing characters and habits of individual species of Myxomycetes during the entire vegetative period have rarely been reported in the literature. This is due principally to the fact that most of them vegetate within their substrata (Lister 1925), making it difficult to obtain their plasmodia for observation and study.

Hemitrichia vesparium (Batsch) Macbr. is such a species. It vegetates unseen within its substratum, where it also sclerotizes, and comes to the surface only to fruit. Although this species is commonly collected in the fruiting stage, there is no record in the literature to indicate that its true plasmodium has previously been recognized in the field.

The outstanding characteristic feature of the plasmodium is its stable, definitely black color. This color, unusual for slime-mold plasmodia, together with certain other constant features, makes specific identification possible before fruiting occurs. While taxonomic characters have not hitherto been reported for the vegetative stage of any species, it is believed, in view of the studies and observations made on this particular species, that they may be found to be rather widespread.

This report is based on observations made on forty-odd plasmodia of *H. vesparium* which were isolated and cultured during the past six years. These plasmodia and most of their subcultures fruited, bearing sporangia typical of the species. The sporangia were verified as to species by Mr. Robert Hagelstein, Honorary Curator of Myxomycetes at the New York Botanical Garden, who examined typical specimens of the sporangia submitted to him.

LITERATURE

The reports thus far published on *H. vesparium*, with one exception, are based on spore sowings made in the laboratory. They show that four-year-old spores have been found to be viable (Gilbert 1929); that the plasmodium is parasitic on fungus mycelia (Howard and Currie 1932); that the plasmodium requires no light in order to fruit (Gray 1938); and that the swarm-cells thrive well in pine wood decoction and can withstand temperatures as low at 2° C without encysting immediately (Smart 1938 a, b). Only two investigators have reported on the color of the plasmodium: Gray 1938, Carr 1939.

MATERIALS AND METHODS

The plasmodia of this species were obtained mostly by culturing decaying wood and leaves. Collections of such material were made in New York State (including Long Island, Gardiner's Island, and Staten Island), New Jersey, Pennsylvania, and southern Vermont. The plasmodium was isolated from material collected in all of these regions. Several plasmodia were grown from laboratory-developed fruiting bodies, the spores of which were sown on blotting paper which had previously been used as substratum. Numerous subcultures were also grown, some of them with other species in order to observe communal behavior. All of the plasmodia were cultured on white blotting paper, which was obtained in large desk-size sheets, watermarked in squares. The sheets were then cut to the desired size and shape as needed. Tap water was used as the source of moisture.

The method employed for isolating the plasmodia from their original substrata was to place a suitably sized disk of blotting paper, cut in halves, in a petri dish and moisten it with water. A selected piece of wood or leaves, recently collected and still moist, if possible, was then laid on the paper, and the culture kept saturated by subsequent additions of water as required. As soon as the plasmodium had left its original substratum, the latter was removed. Plasmodia thus isolated were given nutrient every few days, and were cultured and subcultured both in petri dishes and in jars.

When plasmodia were grown in jars, the method used was an adaptation of that employed by Camp (1936) in culturing *Physarum polycephalum*. Small glass-covered jars were preferred, which measured 3½ inches high and 2¾ inches in diameter. For starting these cultures, a portion of blotting paper containing the plasmodium was transferred from a petri dish culture to a jar that had been lined around the inside wall with two-inch squares of blotting paper and filled to a depth of about one inch with water. Such cultures provided the plasmodium with a continuous, partially submerged circuit or pathway around which it might travel progressively without inhibition, and also insured a constant supply of moisture.

Nutrient consisted of pulverized rolled oats (either the "Quick" or regular kinds), tropical fish food, and wheat germ. These were sifted on the cultures through a fine mesh sieve, in front of the plasmodial "fans." Fish food was used mostly for young plasmodia and for plasmodia which had become somewhat sluggish. It was also found to be good in preparing fresh blotting paper substrata. Usually, plasmodia would not creep onto a fresh piece of blotting paper sprinkled with oats until the second or third day, by which time the oats had become somewhat gelatinous. When fish food was used instead of oats, this time was generally reduced to a few hours. Oats, however, was decidedly the preferred nutrient, and was ingested readily later on as particulate food, after the plasmodium had become established

on a fresh piece of paper. Although no such definite reaction was noted in connection with wheat germ, it was continued in use in community cultures, along with oats, since plasmodia of some species thrive on it.

No effort was made to keep the cultures sterile, because it was felt that more natural results could be obtained if conditions approximated more closely those existing in nature. A given piece of blotting paper was therefore continued in use as substratum as long as it remained sufficiently firm, even though mold, bacteria, and other organisms were abundant in the culture. The surface was cleaned every week or ten days either by peeling off with tweezers the top layer of the paper or by flushing the culture with a siphoned stream of water, care being taken to avoid injuring the plasmodium.

CHARACTERS AND HABITS OF PLASMODIUM

The plasmodium is of medium size as compared with those of about fifty other species also grown in culture by the author. It is neither as large as *Physarum polycephalum* nor as small as several others cultured. This applies to the size of the largest veins as well as to the aggregate amount of protoplasm in a plasmodium which has attained its maximum growth.

As cultured, this species shows an ever changing "fan" pattern that is common among slime-molds, but the posterior veins are often looped (fig. 1). When young, it is frequently seen in a twin-fan pattern (fig. 2). The two fans are usually of equal size and are also connected posteriorly by looped plasmodial veins. This pattern may persist for as long as two days, when the fans may merge, or separate entirely. All of the twin-fan patterns observed were remarkably alike in form and size, and they were seen often enough to suggest the term "recurring pattern."

The plasmodial veins of this species vary greatly in diameter, although during the first few weeks they are usually of about equal size. Typical large veins consist of a main black-colored channel, flanked on either side by a thin, hyaline pseudopodial "ruffle" with darkened areas (fig. 3). These ruffles are always present throughout the life of the plasmodium, except when it is very young or very old. In a young plasmodium and in the smallest veins of an older one, the ruffles may be expressed merely as nodes or buds scattered along either side; in old plasmodia—those about ready to fruit—the ruffles have been withdrawn. The protoplasm is characterized by the presence of conspicuous, round, white transparent vacuoles, which are visible under a hand lens. They travel along with the protoplasm, which streams in one direction for about 50 seconds and then reverses its flow for about 45 seconds. The plasmodium, as a whole, advances at the rate of about an inch in three hours. These rates are about average for the slime-molds in general.

The black color of the plasmodium is derived from numerous black pigmented granules. These flow back and forth with the streaming protoplasm,

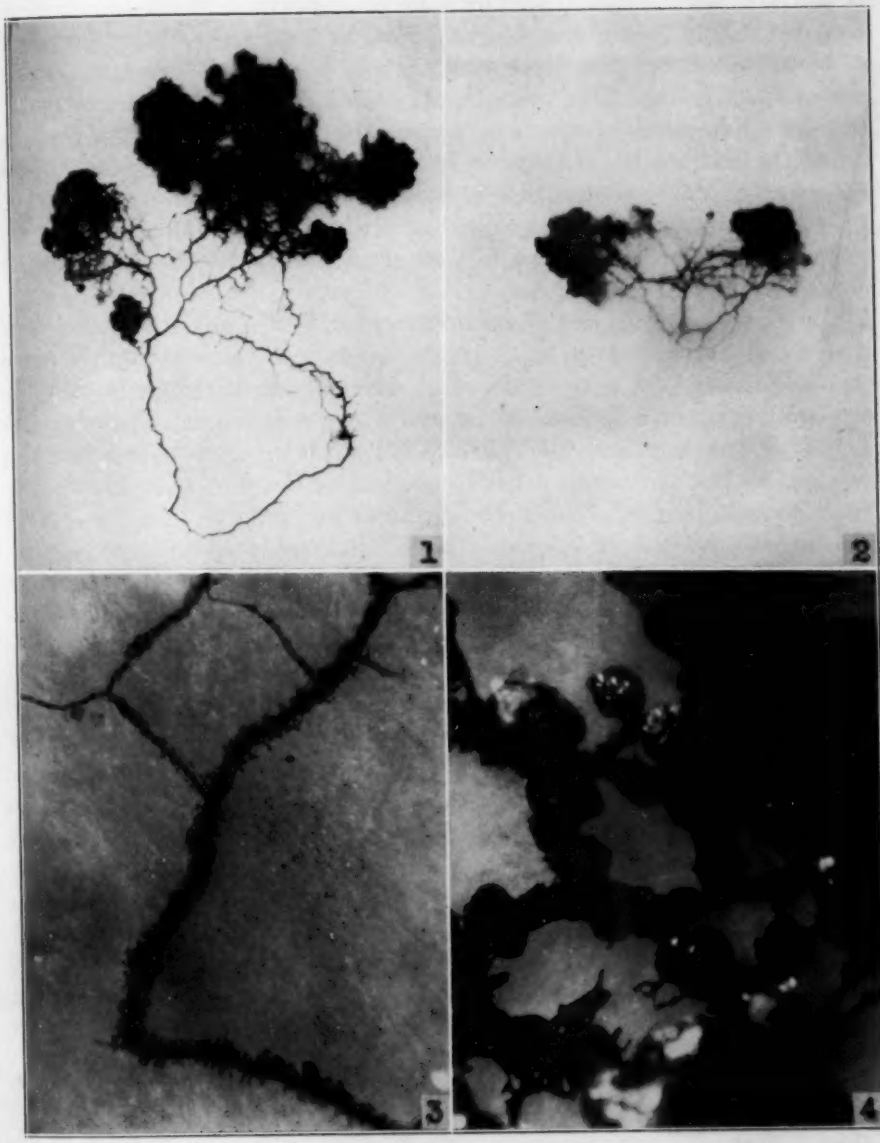

FIG. 1. Vegetating plasmodium, showing a normal variation in looping of posterior veins. ×3. FIG. 2. Recurring pattern. ×3. FIG. 3. Typical veins of a vegetating plasmodium, showing characteristic pseudopodial ruffles. ×30. FIG. 4. Plasmodial veins clumping to fruit. ×33.

being much more abundant in the main channels than in the hyaline pseudopodial ruffles. In these ruffles they are sparse at first, but wherever a number of them become temporarily lodged that area appears black (fig. 3). As the plasmodium ages, the granules seem to increase steadily in numbers throughout the entire plasmodium. As a result, there is a progressive deepening of the color during the vegetative stage. In this connection, Seifriz and Zetzmann (1935) reported that the deepening of the yellow color in the plasmodium of *Physarum polycephalum* is of the nature of a pH indicator, although they did not mention that the change in color was caused by the presence of pigmented granules.

In the field, the plasmodium is not found in places which would suggest that it requires an abundance of moisture; yet in culture it requires more than do plasmodia of those species most commonly grown in the laboratory, especially large ones such as *Physarum polycephalum* and *Fuligo septica*. This is particularly noticeable when the substratum begins to dry out. The two species just mentioned generally remain on the surface, while *H. vesparium* creeps into the substance of the paper and remains there until more moisture is provided. An exception to this habit occurs when the black plasmodium is nearing the fruiting stage, at which time it likewise remains on the surface. This abundance of moisture is evidently required to protect the hyaline pseudopodial ruffles against drying out, for the central vein channels were usually not covered by a film of water as were the ruffles. Keeping the cultures very wet also enabled the plasmodium to make more rapid growth. This species was never observed to creep up the bare sides of glass culture dishes as do the large yellow plasmodia of other species.

The vegetative stage of *H. vesparium* generally lasted from four to twelve months, seven being about the average. Plasmodia which were subcultured several times tended to take longer to fruit than did those not subcultured. One plasmodium grown from spores and not subcultured fruited in two months. Even this short period, however, was much longer than that recorded by Seifriz and Russell (1936) for *Physarum polycephalum*, which had a sixteen-day cycle in the cultures observed. Although no such specific periodicity was observed in *H. vesparium*, fructification was more certain to occur in this species than in any of the other species cultured.

Besides feeding on prepared nutrients, the plasmodium was also observed to ingest living organisms such as protozoa. Several times, when the plasmodium had crept off a small square of blotting paper in a petri dish into a thin film of surrounding water, various kinds of microscopic organisms swarmed and darted about the unusually long hyaline-tipped pseudopods that had just been elongated. After a few minutes a number of the organisms were engulfed by a quick outflow of more hyaloplasm. Several minutes later, black granular protoplasm was seen to stream out and carry the already disintegrating organisms deeper into the plasmodium.

Fragmentation was frequently observed in young plasmodia during the first two months; after that, it seldom occurred. The plasmodial fragments numbered anywhere from two to eight in a given culture at one time. In about a week they gradually coalesced into a single plasmodium, if permitted to, and then in another week or so fragmentation might recur.

In community cultures, the plasmodium was never observed to harm or interfere with a plasmodium of any other species with which it was associated. A small white plasmodium, however, was found to parasitize it (*H. vesparium*), completely consuming it. Over a three-year period, this parasitic plasmodium has been kept in the active vegetating state only by feeding it the plasmodium of *H. vesparium*, although many other nutrients were also tried. When it has no black plasmodium, it sclerotizes. Since parasitism among Myxomycetes themselves seems to be an unrecorded phenomenon, it seems better to prepare a separate illustrated report on the results obtained from culturing these two plasmodia together.

GROWTH OF PLASMODIUM AND SPORANGIAL FORMATION

During the plasmodial stage of *H. vesparium*, four periods of growth were observed: (1) when the plasmodium is very young (scattered nodes along equal-sized veins); (2) when older and making the most growth progress (main channels black, bordered by hyaline pseudopodial ruffles; veins varying in size); (3) when about mature (entire plasmodium, including ruffles, jet black; ruffles appear somewhat fringe-like); (4) when ready to fruit (ruffles entirely absent and main channels swollen and clumped). Although the appearance of the veins is not necessarily uniform throughout a given plasmodium at any one time, the characteristics of one of the four growth-periods enumerated always appear to predominate.

As the plasmodium approaches the fruiting stage, there is a sharp decrease in the amount of moisture and nutrient required. Only a moist substratum need now be maintained instead of a saturated one, and little if any nutrient is needed. Any nutrient that is inadvertently sifted directly upon a jet black plasmodium often causes that portion to turn red, such change of color denoting death in this species.

The amount of moisture present in a culture while the pseudopodial ruffles are disappearing seems to be the principal determinant of how soon afterwards the sporangia begin to rise. Excessive moisture tends to retard fructification and may even cause all or part of the plasmodium to die. Too little moisture inhibits its movement, and if the plasmodium is within the substratum at this time, it lies more or less dormant. Then when moisture is added, it has been observed to well up to the surface of its substratum, in shiny black masses, each a potential cluster of sporangia. At such times no hypothallus is seen, it being left behind within the substratum.

During the prefruiting period, the plasmodium may be either reticulate or in a solid layer. When in a layer, it resembles a thin sheet of shiny black wax in appearance. The veins of a reticulate plasmodium are greatly swollen at this time (fig. 4), and the protoplasmic streaming is very sluggish, as can be detected in the opaque veins only by means of the large transparent vacuoles. Close to a rising sporangial cluster, the streaming is in one direction only—toward and up into the sporangia, with no reversal flow taking place.

Sporangia require about four hours to complete development from the time they begin to rise from the clumped veins. The fruiting process generally occurs between midnight and morning, when no light is present. Although Gray (1938) did not mention this nocturnal habit for his plasmodia of this species, he did state that they required no light in order to fruit.

Fructification was never observed to occur as a result of injury to the plasmodium, lack of food, dehydration, or exposure to bright light. The plasmodia fruited, it seemed, only after a certain degree of maturity or aging had been attained. Camp (1937) reported that *Physarum polycephalum* could be induced to fruit at any time simply by withholding food from it. Such treatment of *H. vesparium* resulted only in a perceptible decrease in the size of its plasmodium. Seifriz and Russell (1936) stated that nutrition and toxic substances alone seemed to have a possible influence on the fruiting of *P. polycephalum*. They reported further that "the growth rhythm is believed to be a definite protoplasmic quality, which requires certain as yet unknown conditions in order to express itself."

Subcultures made from a parent plasmodium that was jet black and nearly ready to fruit, usually bore fruit about the same time as did the parent plasmodium. Seifriz and Russell (1936) also reported this fruiting habit for *P. polycephalum*.

All of the fruiting bodies which developed in culture were either one of the two colors reported by Gilbert (1927) for this species, namely, metallic bluish-black, or brick-red. The brick-red color predominated. Whether the amount of moisture in a culture had any relation to the color of sporangia produced was not determined, but at times this seemed probable.

FORMATION OF SCLEROTIA

The factors reported in the literature as causing Myxomycetes to sclerotize are: lack of food, dehydration, and cold. The last two were the only ones observed to cause the plasmodium of *H. vesparium* to sclerotize. Lack of food, as has just been mentioned, only resulted in a decrease in the size of the plasmodium.

In most instances, sclerotia of this species formed as a result of dehydration. Unlike many larger species, which sclerotize in a heaped mass on the

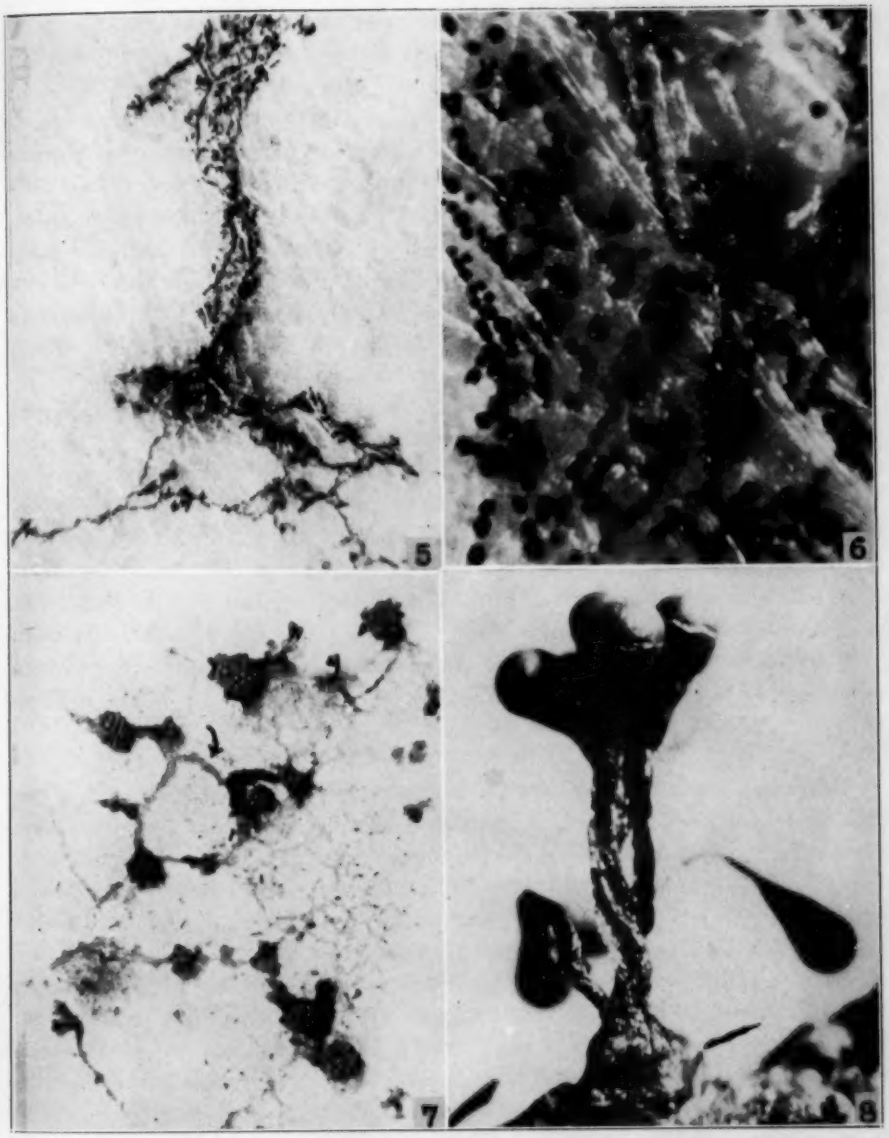

FIG. 5. A sclerotized plasmodium between blotting paper and glass. × 33. FIG. 6. Middle section of figure 5 enlarged. × 150. FIG. 7. Portion of a culture that has fruited, showing dorsal view of sporangial clusters and the red residue left in former vein tracks when the plasmodium fruited. × 3. FIG. 8. Typical sporangia. × 30.

surface of their substratum when dehydration occurs, *H. vesparium* sclerotizes within its substratum (or beneath it) in rows of black bead-like cysts (figs. 5 and 6). This formation results from the breaking up of the plasmodial veins *in situ*.

The cysts are generally round or ovoid in shape when fresh, and range in size from 10 to 40 μ. If formed within blotting paper, a large sclerotium resembles a light-colored blot of ink when viewed from either side without a lens, the individual cysts not being visible. A small sclerotium can sometimes be located only by holding the paper up to the light. When the sclerotium forms beneath the blotting paper, next to the glass, the rows of cysts show up plainly under low magnification. Young cysts that were formed as a result of dehydration required only a day or two to excyst after being moistened, while one-year-old cysts required from 48 to 60 hours.

Exposure to cold caused the plasmodium to sclerotize even when it was covered with water. When subjected suddenly to a temperature of 40° F., the plasmodium sclerotized in about 12 hours. This change occurred so quickly that the original pattern of the plasmodium was retained and a low magnification was required to show that encystment had taken place. Some of the cysts became a transparent red in color and could not be revived, but those that remained black usually could be reactivated into normal plasmodia within a day or so.

Plasmodia that developed from reactivated sclerotial cysts were always black and bore typical sporangia (fig. 8).

PLASMODIAL COLOR

Although black was the only color observed either in the field or in the laboratory for *H. vesparium*, this color does not seem to be mentioned in the literature for this species or for any other vegetating Myxomycete, and inquiry among students of these organisms revealed no one who had previously seen a black plasmodium. In a few instances taxonomists have listed some shade of black as the plasmodial color of a few species, but such color references apparently are not meant to apply to the vegetative stage—as is generally interpreted—but to the prefruiting stage (that which immediately precedes fructification). Macbride (1922, p. 286) states that the colors listed in *The North American Slime-Moulds* refer to the prefruiting stage unless otherwise stated. Hagelstein makes a similar statement regarding colors of vegetating plasmodia, in a personal communication (1938). Since Macbride lists the color of *H. vesparium* as deep red (l. c., p. 262) and does not specify the stage to which it belongs, he, and others who also list some shade of red for the plasmodial color of this species, must therefore mean the color red to refer to the prefruiting stage, and not to the vegetative stage as was concluded by Gray (1938) and Carr (1939).

Gray reported that his plasmodia of this species, which he obtained by sowing spores on corn agar, were pearly white. He stated, however, that they did not grow large, that fruiting was sporadic, and that in no case was an entire plasmodium utilized in sporangial formation. A few of the black plasmodia that I have grown from spores on blotting paper and not transferred or subcultured had a tendency to be somewhat erratic in their fruiting habit in that the plasmodial fragments sometimes remained separate and fruited at different times. In each instance observed, however, all of the protoplasm in a given plasmodial unit was utilized when fructification occurred. This was generally true, also, of plasmodia obtained from material in the field, which, like those grown from spores, sometimes formed five-inch fans when given sufficient space and fed carefully.

It may be pointed out, in connection with Gray's reporting a different color for the vegetative stage of this species, that according to the literature the use of prepared media in culturing spores of Myxomycetes has not proved to be a very reliable source from which to determine colors of vegetating slime-mold plasmodia. Pinoy (1908) reported that he obtained yellow plasmodia as well as blackish-purple ones when he sowed spores of *Didymium nigripes* on flaxseed gelatin. Gray (1938) and Kambly (1939) obtained yellow and white plasmodia, respectively, by sowing spores of *Fuligo septica* on corn agar. On the other hand, plasmodia of *H. vesparium*, according to my observations, were always black, whether observed in the field, isolated from natural substrata on blotting paper, or grown from spores on blotting paper.

Still another color has been reported for this species. Carr (1939), after observing a single plasmodium on wood cultured in a moist chamber for four months and then finding fruiting bodies of *H. vesparium* on the wood, concluded that the yellow plasmodium had borne the sporangia of this species. The evidence is inconclusive. Many times the author has also had sporangia of this species develop on decaying wood cultured in a moist chamber, when the only visible plasmodium was a yellow one. But when such pieces of wood were cultured with a view to isolating all Myxomycete plasmodia present in the wood, as many as four or five other plasmodia, all differently colored, were frequently obtained; that of *H. vesparium* (always black) was often one of them and the only one that produced typical fruitings of this species when grown in isolated cultures. It is therefore doubtful that *H. vesparium* ever has a yellow plasmodium.

Regarding the red color listed by Macbride and other taxonomists as the prefruiting color of *H. vesparium*, this color was not observed in any of the black plasmodia preceding nor during their fruiting period. Sometimes, when viewed by reflected light, the plasmodium had a faint red tinge shortly before fruiting time. This was thought to be due to the presence of extraneous material that was beginning to be cast off, for as the jet black protoplasm

flows into the newly forming fruiting bodies, it leaves a red residue behind in the vein tracks (fig. 7, *arrow*). When fresh, this red residue so closely resembles living plasmodium in consistency that microscopical examination under good light was necessary in order to determine its true nature. Because of this close resemblance, it is wondered whether the appearance of this red residue at fruiting time might account for the statements of Macbride and others that the prefruiting color was red. It would undoubtedly show up more clearly than would black on dark, decaying vegetation. It might even account for Gray's observing (1938) that his pearly white plasmodia of this species turned red at fruiting time but that this color change did not occur until after sporangial delimitation had begun. To determine this point, further observation seems necessary.

Although color as a taxonomic character of vegetating slime mold plasmodia has not yet been recognized, it may not be without significance in helping to determine species of Myxomycetes before fruiting occurs. The consistency with which *H. vesparium* has maintained its color over such a long period of time affords evidence in this direction. Kambly (1939) believes that it is necessary to know the factors influencing color before it can be accepted as a taxonomic character. When color has been found to be constant for a given species, however, it seems logical to recognize its taxonomic value without waiting to determine what causes it.

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SUMMARY

1. The plasmodium of *H. vesparium* can be identified specifically before fruiting occurs and is easily cultured to maturity. It is characterized by a definite black color.

2. Fragmentation occurs frequently during the early life of the plasmodium.

3. The plasmodium has a recurring twin-fan pattern. Posterior veins are often looped.

4. Sclerotia are formed within the substratum in rows of bead-like cysts.

5. The plasmodium is completely parasitized in culture by the plasmodium of another Myxomycete (species as yet undetermined).

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MACROSPOROGENESIS, FERTILIZATION, AND EARLY EMBRYOLOGY OF TARAXACUM KOK-SAGHYZ[1]

H. E. WARMKE

With the introduction of seed of the Russian Dandelion, *Taraxacum kok-saghyz* Rod., into this country early in 1942, and with plans for its extensive cultivation as a domestic source of rubber, it became important to know something more of the cytology and breeding behavior of this species.

The common dandelion, *Taraxacum officinale*, and other species have long been known to reproduce by apomixis (Raunkiaer 1903; Murbeck 1904; Juel 1904, 1905; Sears 1917, 1922). In these forms there is a failure of the first meiotic division, and the second division gives rise to a dyad of macrospores with unreduced nuclei, rather than the usual tetrad with reduced chromosome number (Osawa 1913; Rosenberg 1927, 1930). One of the unreduced cells degenerates (usually the micropylar one), and the other undergoes mitotic divisions to form an eight-nucleate, but diploid, embryo sac. The unreduced egg cell begins to divide, without the stimulation of fertilization, to form a completely vegetative embryo and seed.

With apomixis known to occur in the genus, it was important to ascertain whether the material with which we were working was also apomictic, or whether it was sexual, before entering into selection and breeding experiments. The Russian workers, Poddubnaja-Arnoldi and Dianowa (1934), in a study of several members of the genus, reported *T. kok-saghyz* to be a diploid with chromosome numbers of $n = 8$; $2n = 16$. They further reported that this species is sexually reproducing and highly self-sterile. Our results confirm and extend the findings of these workers.

MATERIALS AND METHODS

Seeds were received on May 16, 1942, from Dr. E. W. Brandes under the label, *Taraxacum kok-saghyz*, plant quarantine number 143960. These were a portion of the seeds which reached this country from Russia by air express. They apparently were from unselected field-grown plants, for in addition to showing extreme variation in size, vigor, and leaf shape, they also contained contaminations of the common dandelion.

Root tips were prepared according to the section-smear technique (Warmke 1941). Buds and flowers were fixed in Rollin Carnoy, sectioned at 10–20 microns according to the stage, and stained with the Feulgen reaction for fertilization and iron hematoxylin for other stages.

[1] Cooperative project with Dr. E. W. Brandes, Pathologist in Charge, Rubber Plant Investigations, U. S. Department of Agriculture.

CHROMOSOME NUMBER

Root tips were taken from plants growing in four-inch pots in the greenhouse; chromosome counts showed the diploid number to be 16 (fig. 1, a and b). No variation from this number has been observed. The chromosomes are of medium size, ranging from approximately 2 to 4 microns in length in the roots, and fix and stain well. One pair of chromosomes bears prominent satellites (fig. 1, b).

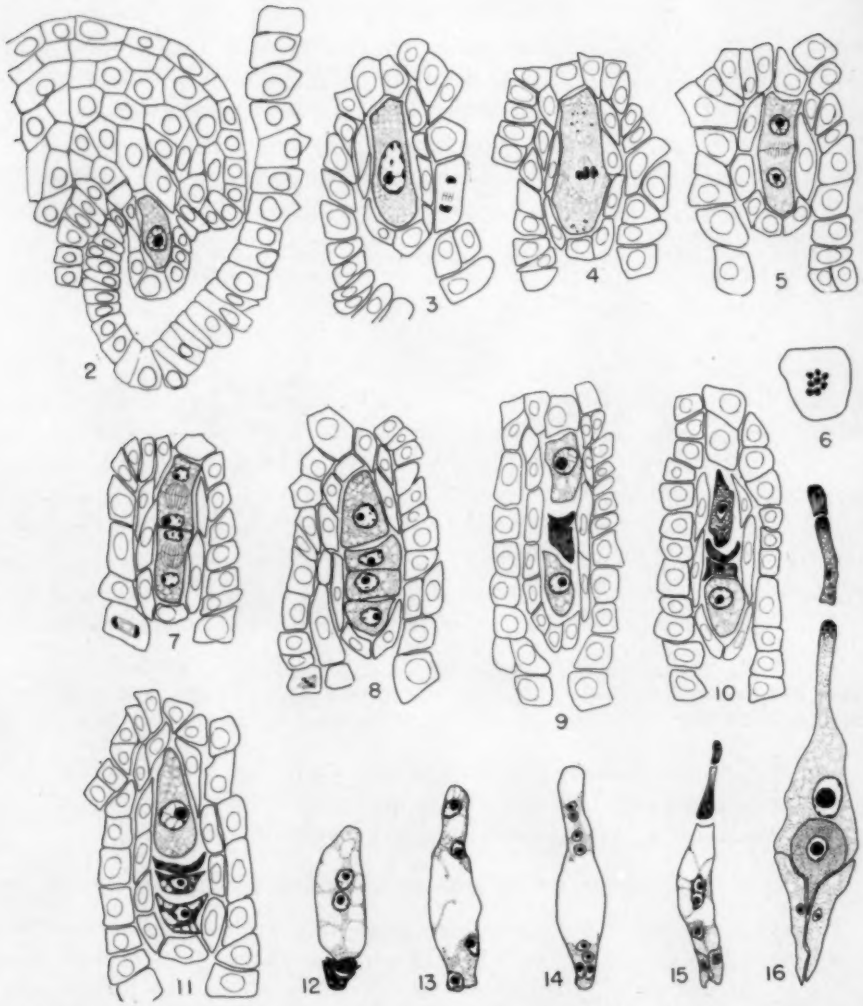
FIG. 1. Photomicrographs of somatic chromosomes from root tips of *Taraxacum koksaghyz*. The diploid chromosome number is 16. Note one pair of chromosomes with satellites (fig. 1b). × 1400.

The haploid chromosome complement was studied at first meiotic metaphase in macrospore mother cells (fig. 4) and in pollen mother cells (fig. 6). In all cases the reduced chromosome number was found to be 8.

DEVELOPMENT OF THE MACROGAMETOPHYTE

The ovule is solitary and anatropous. The macrospore mother cell arises from a hypodermal cell and may be distinguished early by its greater size and more intense staining reaction (fig. 2). Surrounding the macrospore mother cell is a single layer of nucellar cells, which is soon overgrown and surrounded, except for the micropyle, by a single integument. The macrospore mother cell increases further in size (fig. 3) and then undergoes the first meiotic division (fig. 4). The chromosomes are arranged in a regular manner as bivalents on the metaphase plate, with no indication of univalents or laggards.

At the end of MI a cell wall is laid down (fig. 5) to form the usual dyad. The second meiotic division follows quickly, and cell walls are laid down to form a linear tetrad of macrospores (figs. 7, 8). The chalazal macrospore tends to be larger than the others (fig. 8) and usually becomes functional,



FIGS. 2–16. Meiosis and macrogametophyte formation in *T. kok-saghyz*. FIG. 2. Young ovule showing macrospore mother cell before overgrowth of the integument. × 540. FIG. 3. Older macrospore mother cell. × 540. FIG. 4. First meiotic metaphase; eight bivalent chromosomes are present. × 540. FIG. 5. Late telophase of first meiotic division with dyad of cells being formed. × 540. FIG. 6. MI in pollen mother cell showing 8 chromosomes. × 840. FIG. 7. Late telophase of second meiotic division. × 540. FIG. 8. Linear tetrad of macrospores. × 540. FIG. 9. Middle two spores of tetrad tend to degenerate early. × 540. FIG. 10. A rare case, where micropylar macrospore is functional. × 540. FIG. 11. The more usual condition, where the chalazal macrospore becomes functional, and the others degenerate. × 540. FIGS. 12–14. 2-, 4-, and 8-nucleate stages in the development of the macrogametophyte. × 270. FIG. 15. Young 7-celled macrogametopyte. Note union of the polar nuclei and beginning of degeneration of antipodals. × 270. FIG. 16. Mature macrogametophyte. × 270.

while the three other macrospores degenerate (fig. 11). There is a strong tendency in many of the ovules, however, for the middle two cells to degenerate quickly and for both the micropylar and chalazal ones to persist and enlarge (fig. 9). One of these eventually gains the ascendency and the other degenerates, for twin embryo sacs have not been observed. The basal cell usually becomes functional, but figure 10 shows a case where the micropylar cell seems to have become the functional macrospore.

This process appears to be an orderly and complete meiotic division and to have resulted in the formation of four reduced macrospores. It is quite distinct from the abortive process in the common apomictic species, in which two unreduced macrospores are formed as the end-result of meiosis.

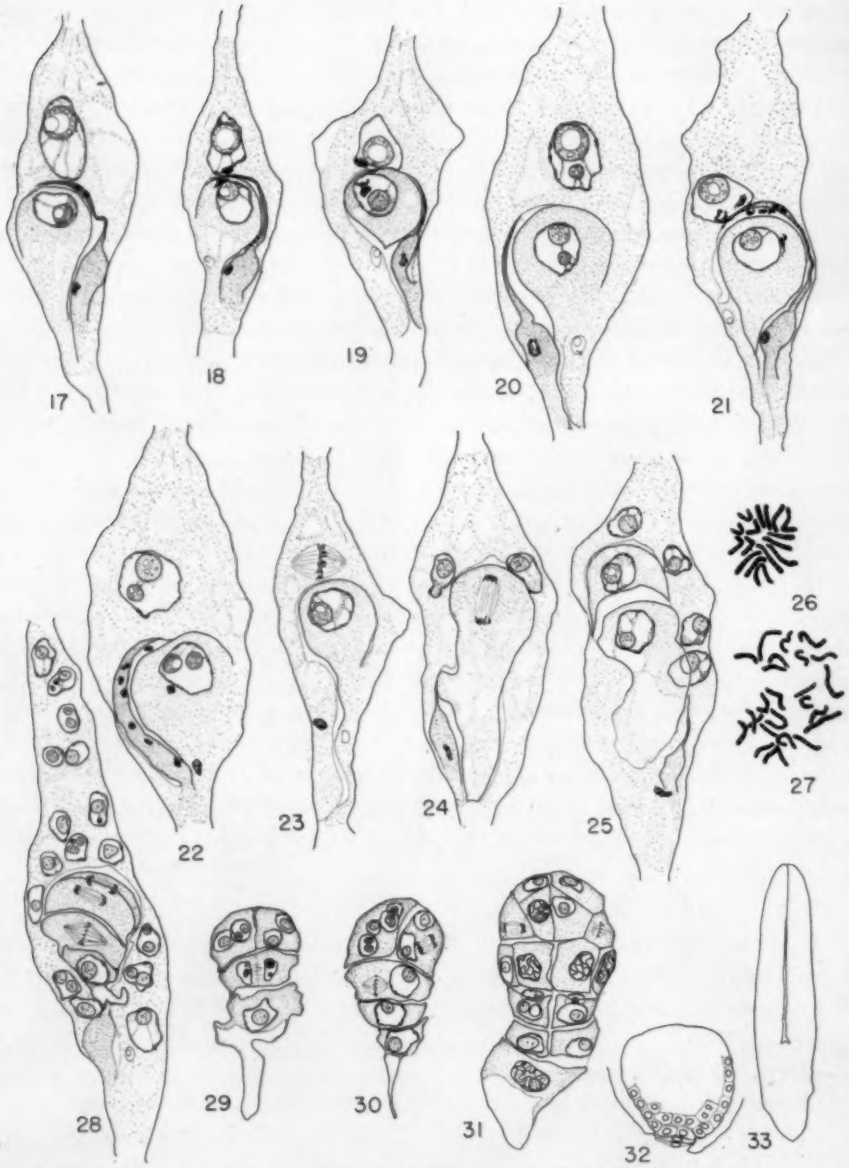
Before the non-functional spores have completely degenerated, the first mitotic division in the formation of the macrogametophyte occurs (fig. 12). The second and third divisions follow in rapid succession, forming four-nucleate (fig. 13) and eight-nucleate (fig. 14) macrogametophytes. During these free nuclear divisions the macrogametophyte increases in size, largely by the process of vacuolation. It is also evident that as the number of nuclei increases, the individual nuclei decrease in size (figs. 12–14).

The two most centrally located nuclei approach each other in the usual manner to become the polar nuclei (fig. 15). Note in figure 15 that these two nuclei, though in contact, are still separated by the nuclear membranes. Cell walls are laid down and soon transform the macrogametophyte into the typical seven-celled structure, consisting of two synergids, an egg cell, a large and vacuolate primary endosperm cell, and three antipodals. These latter become pycnotic and start to degenerate even before the polar nuclei have completely fused (fig. 15). The macrogametophyte enlarges greatly until it reaches its mature size (fig. 16), which represents the condition at flowering.

FERTILIZATION

Fertilization begins about 30 minutes after pollination at 70° F. under greenhouse conditions. This may be compared with 15 minutes reported for this species under slightly different conditions by Poddubnaja-Arnoldi and Dianowa (1934). Our fixations at 10, 15, 20, and 25 minutes failed to show evidence of fertilization.

Thirty minutes after pollination many ovules are seen in which pollen tubes have penetrated the embryo sac (apparently through a synergid) and have wedged in between the egg and primary endosperm cells, separating these structures (fig. 17). Whether this is actually the tip of the pollen tube, or some material extruded from the tube, is not clear. This material is uniform in consistency and appears to be somewhat more dense than the cytoplasm of the surrounding embryo sac. It has a characteristic sickle shape and bears near its base a single small nucleus which stains darkly. It is not cer-



FIGS. 17–33. Fertilization and embryo formation in *T. kok-saghyz*. FIG. 17. 30 minutes after pollination: pollen tube with two male nuclei near its tip within the embryo sac. ×380. FIG. 18. 30 minutes after pollination: male nuclei in contact with nuclear membranes of egg and endosperm cells. ×380. FIG. 19. 45 minutes after pollination: male nuclei within egg and endosperm nuclei. ×380. FIG. 20. 2 hours after pollination: the intensely staining male nuclei have disappeared and are replaced by small extra nucleoli within egg and endosperm nuclei. ×380. FIGS. 21, 22. 45 minutes and 2 hours,

tain whether this is the tube nucleus or the nucleus of the synergid which was penetrated by the pollen tube, although the latter seems more likely, since this pycnotic nucleus always lies approximately opposite the undisturbed synergid nucleus (figs. 18, 19, 20, 21).

With the Feulgen reaction, the large egg and endosperm nuclei take a very light stain, in contrast to the sperm nuclei, which stain very intensely. When first seen, the male nuclei appear to lie within the pollen-tube material (fig. 17) but soon come to lie out of this and in contact with the egg and endosperm nuclei (fig. 18).

Forty-five minutes after pollination the two male nuclei are seen inside the egg and endosperm nuclei; they have increased in size, and have become more diffuse (fig. 19). In favorable preparations male nuclei at this stage are seen to be made up of expanding and uncoiling chromonemata. At two hours, nuclear fusion is complete (fig. 20). The intensely staining chromatin of the male nuclei is no longer in evidence; in its place two small nucleoli have appeared, one each in the egg and endosperm nuclei. The pollen-tube material is still present, as well as the cavity separating egg and endosperm cells.

As noted by the Russian workers, supernumerary male nuclei are not uncommon. Figures 21 and 22 show two such cases. In the first of these a total of 8 male nuclei is present in one embryo sac; while the second must have 10: 8 densely staining ones and two already united with the egg and endosperm nuclei, as indicated by the presence of two nucleoli in each of these nuclei. Figure 22 is a two-hour stage, and the double nucleoli almost certainly indicate previous nuclear fusion, just as they do in figure 20.

The supernumerary sperm nuclei usually do not function, as is indicated by their presence as separate, compact bodies lying in the pollen-tube material or adjacent cavity even after the egg and endosperm have begun mitotic divisions. Figure 21, however, seems exceptional: here two nuclei lie in the tube, three in the adjacent cavity, one against the egg nucleus, and two against the primary endosperm nucleus. The sperm nuclei in contact with the egg and the endosperm nuclei are becoming diffuse. This suggests

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respectively, after pollination: supernumerary male nuclei are occasionally found within the embryo sac. ×380. FIG. 23. 6 hours after pollination: the endosperm nucleus is undergoing its first division. ×380. FIG. 24. 7 hours after pollination: the endosperm is 2-nucleate, and the zygote is undergoing the first division. ×380. FIG. 25. 9-hour stage: the proembryo is 2-celled, and the endosperm is 4-nucleate. ×380. FIG. 26. Metaphase plate from dividing embryo cell showing diploid number of chromosomes, $2n = 16$. ×1400. FIG. 27. Late prophase from dividing endosperm showing triploid number of chromosomes, $3n = 24$. ×1400. FIG. 28. Proembryo 4-celled, and endosperm 16-nucleate. ×380. FIG. 29. 7-celled proembryo. ×380. FIG. 30. 11-celled proembryo. ×380. FIG. 31. 24 hours after pollination: proembryo is made up of 28–30 cells. ×380. FIG. 32. 40–44 hours: young spherical embryo with short suspensor. ×85. FIG. 33. 55–60 hours: the embryo has assumed mature form, with cotyledons, epicotyl, hypocotyl, and root tip. ×25.

that these two male nuclei, instead of one as expected, may be uniting with the endosperm. There is some further evidence that functional polyspermy may occur in connection with the endosperm, in the fact that four or five separate nucleoli may occasionally be observed in a single endosperm nucleus. Since a maximum of one nucleolus is characteristic of each haploid chromosome complement, the presence of four or five nucleoli in endosperm cells may be indicative of extra sets of chromosomes, although no more than the triploid number of chromosomes has ever actually been counted in dividing endosperm.

DEVELOPMENT OF THE EMBRYO AND ENDOSPERM

At approximately 6 hours after pollination, under our conditions, the first division of the endosperm occurs (fig. 23). This is usually followed about an hour later by the division of the zygote (fig. 24). At 9 hours after pollination the proembryo is 2-celled and the endosperm 4-nucleate (fig. 25). Chromosome counts from dividing cells show the embryo to be diploid (fig. 26) and the endosperm to be triploid (fig. 27).

The further development of the embryo follows the "aster type," characteristic of the composites. The "basal cell" (according to the terminology of Souèges) undergoes a transverse division; while the "apical cell" divides in a longitudinal plane. This is illustrated in the 4-celled stage (fig. 28). Here the basal cell has divided to form two daughter cells, one above the other; while the apical cell has divided so that one daughter cell lies behind the other.

The second division of the apical cell is also longitudinal, but at right angles to the plane of the first, as shown by the orientation of the division figures in figure 28. The uppermost half of the original basal cell also divides longitudinally, thus producing a 7-celled proembryo (fig. 29).

The proembryo undergoes rapid division to reach the 11-celled stage shown in figure 30. There are 7 derivatives of the original apical cell and 4 derivatives of the original basal cell. At 24 hours the proembryo has a total of 28–30 cells (fig. 31).

At 40–44 hours the embryo proper has formed a flat ball of several hundred cells, with a short suspensor consisting of 8–12 cells (fig. 32). At about 60 hours the embryo has attained mature appearance with well developed cotyledons, epicotyl, hypocotyl, and root tip (fig. 33).

BREEDING BEHAVIOR

The first flowers were observed August 16 on greenhouse-grown plants; this was exactly three months after sowing the seed. Plants have continued to come into flower throughout the fall and winter, with approximately one-third having flowered by January 1, 1943. During the summer and early fall

none of the plants that flowered set any seed, unless they were mechanically cross-pollinated by rubbing together heads from different plants. After such cross-pollination abundant seed was set, which ripened in about 10 days. The greenhouses were screened against insects; so this behavior indicates a high degree of self-sterility. Even when plants were mechanically self-pollinated by rubbing two flowers from the same plant together, seeds failed to set. Plants of the common apomictic species of dandelion, of course, set seed abundantly without any sort of pollination under identical conditions.

During November and December, however, many *T. kok-saghyz* plants began to set seed without cross-pollination. Some of these heads bore only 1 or 2 seeds while others bore a full set of 50–75 seeds. It would appear that this is an "end-season fertility," induced possibly by temperature, light, or age of the plant. It has not yet been possible, however, to show definitely that this delayed fertility is due to selfing and not to apomixis; but it seems highly improbable that the latter could be the case.

DISCUSSION

Chromosome numbers of $n=8$ and $2n=16$ place *T. kok-saghyz*, along with six or eight other species, as basic diploids in the genus. The great majority of the genus, however, is polyploid. Species with sporophytic chromosome numbers of 24, 32, and 40, representing triploid, tetraploid, and pentaploid forms, respectively, are known. It is of interest that the diploid species, including *kok-saghyz*, are sexually reproducing, while the polyploids are apomictic. This close correlation between polyploidy and apomixis has long been noted but is not yet thoroughly understood. As pointed out by Stebbins (1941) hybridization and polyploidy may be factors in bringing together complementary genes for apomixis and in the production of vigorous sterile or partially sterile types in which apomixis would have a very high selective value.

The reproductive process in *T. kok-saghyz* is completely normal and sexual. On the female side, where sexual reproduction breaks down in apomictic species, two regular meiotic divisions occur to produce a linear tetrad of reduced macrospores. There can be no doubt about the occurrence of fertilization: all stages, from the entrance of the pollen tube to complete union of the male nuclei with egg and endosperm, have been observed. Moreover, when pollination and fertilization do not occur, there is no development of embryo or endosperm. Final proof of the reality of the reduction divisions and of fertilization is afforded by actual chromosome counts, in which the developing embryo has been shown to be diploid (16 chromosomes) and the developing endosperm triploid (24 chromosomes). Presumably the embryo would be diploid and the endosperm tetraploid if this species were apomictic.

These conditions, including ample heterozygosity, low chromosome num-

ber, normal sexual reproduction, and a high degree of self-sterility, are extremely favorable from the standpoint of breeding and selection experiments. With satisfactory methods of assaying the rubber content of individual plants, the breeding of these plants and the selection of strains with improved cultural and rubber-producing qualities should be routine for the experienced plant breeder.

SUMMARY

1. The chromosome number in *Taraxacum kok-saghyz* is $n = 8$; $2n = 16$, placing this species among the basic diploids of the genus.

2. Macrogametophyte formation follows the usual sexual pattern: The macrospore mother cell undergoes two regular meiotic divisions to form a linear series of four reduced macrospores, the chalazal one of which usually becomes functional. As the result of three successive mitotic divisions followed by union of the two polar nuclei and cell wall formation, the classic 7-celled macrogametophyte is formed.

3. Thirty minutes after pollination, pollen-tubes are seen to enter the macrogametophyte. Fertilization follows the normal pattern: one male nucleus uniting with the egg and the other uniting with the primary endosperm nucleus. Supernumerary male nuclei are frequently observed in the embryo sac but usually are not functional.

4. The first division in the endosperm occurs about 6 hours after pollination, and the first division of the egg follows about one hour later.

5. Embryonic development follows the aster type, characteristic of the compositae.

6. Chromosome counts verify the reality of the sexual processes by showing the developing embryo to be diploid, with 16 chromosomes, and the endosperm to be triploid, with 24 chromosomes.

7. During the summer *T. kok-saghyz* is highly self-sterile but cross-fertile. In the late fall and winter it may exhibit considerable end-season self-fertility.

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TWO NEW SPECIES OF HYPERICUM FROM COLOMBIA

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In recent Cuatrecasas collections from northeastern Colombia the following two new species of *Hypericum* were found. They belong to the section *Brathys*, subsection *Eubrathys*, which has been previously treated by Gleason.[1]

Hypericum garciae Pierce sp. nov. Frutex ramis virgatis vel erectis inferne denudatis; folia rigida laxe imbricata, anguste lanceolata, 6–7 mm. longa, 1.3 mm. lata, punctata, margine integerrimo revoluto, nervo medio subtus prominenti, nervis lateralibus nullis; flores solitarii ad apices ramulorum; sepala ovato-lanceolata, 5.8–6.2 mm. longa, 1.5–1.8 mm. lata; petala oblique obovata, apiculata. 10 mm. longa; ovarium ovatum, uniloculare, 3–4 mm. longum, septis nullis, placentis 3 parietalibus; styli 3, 5.5–5.8 mm. longi.

TYPE, *Cuatrecasas & Garcia Barriga 9935*, collected on the Paramo del Almorzadero, Dept. of Santander, in the Cordillera Oriental of Colombia, and deposited in the Britton Herbarium of the New York Botanical Garden. This species resembles closely *H. chamaemyrtus* Tr. & Pl. but differs in having smaller, more strictly ascending leaves, solitary flowers, smaller petals, fewer stamens, shorter styles, and a larger ovary.

Hypericum cuatrecasii Gleason sp. nov. Frutex ramosus ramis inferne denudatis bialatis infra foliorum costam mediam. Laminae subcoriaceae patulae late rotundo-ovatae, usque 7 mm. longae lataeque, aut in ramulis tantum 2 mm. longae, ad basim cordato-amplectentes paulo connatae, 1-nerviae, venulis, reticulatis vix perspicuis. Pedicelli brevissimi, calyce foliis superioribus fere obtecto. Sepala ovato-elliptica obtusa, 5–5.3 mm. longa, 3–3.9 mm. lata. Petala asymmetrica 12 mm. longa. Stamina numerosissima (in flore uno 187 numerata), usque 6.5 mm. longa. Antherae 0.5 mm. longae. Ovarium late ellipsoideum, 3 mm. longum; styli 3, fere recti, crassi, divergentes, 1.1 mm. longi.

TYPE, *Cuatrecasas 10439*, collected on the Paramo de Arcabuco between Arcabuco and Tunja, Boyacá, Cordillera Oriental, Colombia, and deposited in the Britton Herbarium of the New York Botanical Garden. The cordate-clasping leaves, the large number of stamens, and the very short styles set *H. cuatrecasii* apart as unique among the species of the subsection *Eubrathys* known from South America.

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[1] Bull. Torrey Club **56**: 100–107. 1929; Torreya **29**: 137. 1929.

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THE METAPHASE STAGE IN COLCHICINIZED ONION ROOT-TIPS

MICHAEL LEVINE AND SIDNEY GELBER

The lethal effects of X-ray and radium emanations on neoplastic tissues are in a large measure dependent upon the stage of development of the component cells. The dividing cell, it seems, is more vulnerable to these physical agents than the resting ones. The neoplasia of animals present many difficulties to the radium or X-ray therapeutist because of the great variations in the number of dividing cells in such tissues. Arresting cell division in the metaphase stage in neoplastic tissue of animals by the application of colchicine has revealed no constancy in the number of dividing cells, for in the individual tumor DuBilier and Warren (1941) have shown that the maximum effect of colchicine on the Brown-Pearce rabbit epithelioma is attained in 6 hours with a single dose (0.1 mg. per 100 gm. body wt.); with repeated doses the colchicine attains its maximum effect at 12 hours. These results apply to an average for a given dose, yet the response of the individual tumor varies greatly. The results are so variable that these authors believe that a trial of the effect of colchicine and roentgen therapy is impracticable. This is readily understood, for in transplantable tumors the rate of growth may vary with the transplant, the host, and other factors still unknown.

The ready availability of dividing onion root-tip cells and the ease with which this tissue may be observed has led us to the study of the effect of X-ray on this tissue affected by colchicine. The arrest of the mitotic changes in metaphase exposes the cells at a stage when X-rays are supposed by many to be most effective in destroying them. It accordingly became necessary to determine at what time after treatment colchicine-treated onion roots present the greatest number of dividing cells in metaphase.

The following is a report on the number of cells in metaphase stage found in the root tips of *Allium cepa*, var. Yellow Globe, after exposure to .01 per cent colchicine solution. The regularity with which the root-tips of the onions hypertrophied after treatment with the solution of colchicine used seemed to indicate that the maximum changes occurred in about 48 hours. Cytological preparations of this tissue showed however, that the largest number of dividing cells were to be found at much shorter exposures. A systematic study was made of root tips exposed to a uniform solution of colchicine (.01 per cent) from 6 hours to 6 days. The number of resting cells and cells in the metaphase stage was counted in four root-tips selected at random after each of the following periods of exposure: 6, 9, 12, 24, 36, 48, 72, 96, 120, and 140 hours.

The effect of colchicine as it is now well established, is to arrest the mitotic division in the metaphase stage. While it was first thought that colchicine increased the number of division stages, Ludford (1936) showed that it arrested the cell in its metaphase division stage so that the number of dividing cells appeared to increase.

Ludford showed that .00001 per cent solution is effective on mitosis in some animal tissue. Levan (1938) studied the effects of various concentrations of colchicine at time varying from 7 minutes to 72 hours after the treatment. Concentration of .0055 per cent, he believed, was sufficient to cause disturbance of the spindle figure. Levine and Lein (1941) showed that a .001 per cent solution of colchicine was sufficient to arrest the elongation of roots of the onion. Levan further observed that the threshold value for affecting mitosis in roots of *Allium cepa* with colchicine lies between .005 per cent and .01 per cent solutions after an exposure of 4 hours. We selected the larger dose (.01 per cent solution) for our studies.

METHODS AND MATERIALS

The onions were drawn from regular shipments made to this institution for dietary purposes. These were selected from large numbers for uniformity of size, weight, shape, and color; the genetic constitution of the species was unknown.

The resting bulbs were placed on top of cylindrical vessels of 240 cc. capacity, filled with cool fresh water daily, so that the root end of each bulb was constantly immersed. The vessels were coated on the outer surface with a dense opaque material so as to exclude light. The bulbs were permitted to rest in water until a sufficient number of roots had been formed. After a period of from 7 to 10 days bulbs with a fair number of roots (15–20) were transferred to similar vessels filled with a .01 per cent aqueous solution of colchicine. These bulbs were labeled and placed in a moderately cool part of the laboratory with constantly subdued light. To obtain as much diversity in cell counts as possible, roots from four different bulbs were used in each test. The onions used were divided into four series each comprising from 12 to 20 bulbs. The root-tips were examined first after exposure to the colchicine solution for 6-, 24-, and 48-hour periods. Three additional groups of onions with many controls were selected from those exposed 9, 12, 36, 72, 96, 120, and 140 hours to the colchicine treatment. These were studied in the following order: 36, 120, and 140 hours of exposure, 9, 72, and 96 hours of exposure, and 12 hours of exposure.

After a given exposure to the colchicine was made, the root-tips from each onion were fixed according to Warmke's (1935) method; although fixation for greater periods than those suggested by Warmke was found necessary for root-tips exposed for long intervals. The root-tips were

stained in aceto-carmine and chlorazol black E, as described by Nebel (1940). The latter dye intensifies the stains and makes the cell- and chromosome-count more reliable. More than 4000 cells were counted in each series after exposure to colchicine for a given time, and the percentage of cells in metaphase stage was calculated. Control bulbs were grown in water at the same time for from 7 to 15 days, but not exposed to colchicine; the water was changed daily. The roots of these bulbs were fixed and smeared in the same way. More than 4000 cells were counted from this group.

CELL COUNTS IN UNTREATED BULBS

The untreated roots were a bright and glistening white. The cells of four were counted; of 4112 cells there were 3963 in resting or prophase stages and 149 in metaphase, 3.6 per cent of the total counted (Table 1). Three of the counted root-tips contained an approximately equal number of cells in metaphase stage. One root contained 58, an extreme divergence from the average. This may indicate the selection of a shorter, younger root, although the effort has been made to select for the count roots of equal length. The extreme percentages are represented in the graph (fig. 1) by broken lines, while the black line represents the average percentages of metaphase stages based on the figures given in the accompanying table.

CELL COUNTS IN TREATED BULBS

In table 1, the counts have been made from bulbs exposed to .01 per cent colchicine for 6, 9, 12, 24, 36, 48, 72, 96, 120, and 140 hours. During long exposures the colchicine solutions were not changed but fresh solution was added as required. The tips were removed, fixed, and smeared by the methods mentioned above. The cells in resting and metaphase stages were counted. Root-tips exposed to .01 per cent colchicine for 6 hours have a uniform number of metaphase stages, all but root 3F which has only 55 cells in metaphase stages against 73 in the other roots recorded (see table 1). The average percentage (6.7) of cells in the metaphase stage has almost doubled as a result of the exposure. At any time after exposure the departures from the average are approximately equal, as shown by fig. 1. In the 9-hour series, 4320 cells were counted, of which 405 were in metaphase, 9.38 per cent of the total cells counted. The variation in the number of cells in metaphase is small. The tendency to an increase of the number of metaphases is now apparent, as shown in table 1. This tendency became obvious when the counts for the different periods of exposures were arranged chronologically. Among roots exposed for 12 hours, the difference between roots 2T and 5T is 35 cells in metaphase; approximately equal to the difference between extremes in the untreated roots. The number of metaphase stages in this series totals 490 in 4195 cells counted (11.6 per cent) as against 405 in 4320 cells

TABLE 1. *Number of cells in metaphase in untreated roots and in roots treated with .01 per cent colchicine*

Exposure	Root	Cells counted	Cells in metaphase	Per cent cells in metaphase
None	3	1026	26	2.53
	1B	1017	34	3.34
	2B	1043	58	5.56
	3B	1026	31	3.02
		4112	149	3.62
6 hours	2F	1016	73	7.18
	3F	1026	55	5.36
	4F	1019	71	6.96
	5F	1021	76	7.44
		4082	275	6.74
9 hours	2H	1109	107	9.64
	3H	1036	105	10.13
	5H	1143	96	8.39
	6H	1032	97	9.39
		4320	405	9.38
12 hours	2T	1149	143	12.44
	3T	1031	116	11.25
	4T	1011	123	12.16
	5T	1044	108	10.34
		4195	490	11.68
24 hours	1E	1034	156	15.08
	2E	1232	204	16.55
	3E	1022	156	15.45
	4E	1010	121	11.98
		4298	637	14.88
36 hours	5X	1210	101	8.34
	6X	1170	104	8.88
	9X	1045	102	9.76
	10X	1041	116	11.14
		4466	423	9.47
48 hours	2C	1057	85	8.04
	3C	1097	96	8.75
	4C	1002	75	7.48
	6C	1026	80	7.79
		4182	336	8.03
72 hours	6Z	1109	60	5.40
	7Z	1062	61	5.74
	8Z	1117	84	7.52
	10Z	1123	70	6.23
		4411	275	6.23
96 hours	3V	1110	39	3.51
	4V	1053	23	2.18
	5V	1039	45	4.33
	7V	1084	53	4.88
		4286	160	3.73
120 hours	2Q	1195	46	3.84
	5Q	1028	33	2.99
	6Q	1050	28	2.66
	7Q	1235	55	4.45
		4508	162	3.59
140 hours	3L	1125	33	2.93
	5L	1023	24	2.34
	6L	1034	53	5.12
	8L	1081	16	1.38
		4263	125	2.93

counted (9.3 per cent) after the 9-hour treatment and 275 in 4082 cells counted (6.7 per cent) after the 6-hour treatment. It appears that some

FIG. 1. The percentages of cells in metaphase in the root tips of *Allium cepa*; untreated and exposed to a solution of .01 per cent colchicine for from 6 to 140 hours. Middle line indicates average percentage; upper broken line indicates maximum percentage; lower broken line indicates minimum percentage.

roots attain the maximum number of metaphase stages after this exposure, as root 2T.

Of a total of 4298 cells counted in root-tips exposed for 24 hours to the same concentration of colchicine 637 cells were found in metaphase. This is an increase of 11.2 per cent metaphases over the number counted in the untreated root-tips and an increase of 3.2 per cent over the 12-hour-treated root tips. The 24-hour exposure seems to yield the maximum effect with .01 per cent solution of colchicine. From a study of figure 1 it is clear that the greatest extremes, in the number of metaphases also occur here. It appears that it represents for some roots the critical exposure, for in the following treatments a definite decrease in the number of metaphase stages is met, as shown by table 1. After the 36-hour exposure there are fewer metaphases; from 637 of 4298 cells for the 24-hour period to 423 of 4466 cells (9.4 per cent). The extremes are represented by 101 metaphases in 1210 cells counted in root 5X and 116 metaphases in 1041 cells counted in root 10X, a difference of 2.8 per cent. In this series the results may be interpreted as a toxic effect on cells in the resting stages. Some of the cells in the metaphase stage may have completed their nuclear reconstruction, or some may have degenerated.

In root-tips exposed for 48 hours to .01 per cent colchicine there are still fewer metaphases than in those exposed for 36 hours. The continued exposures observed after 72, 96, 120, and 140 hours showed a gradual decline in the percentage of cells in metaphase, until in the latter exposures we have an average percentage of metaphases slightly lower than that of the untreated roots (see graph). From the 96-hour to the 140-hour exposures the differences between extremes are approximately uniform; especially is this noted in the 120-hour-treated roots. The number of metaphases counted in roots exposed for 96 and 120 hours are 160 and 162, respectively, from counts of 4286 cells in the 96-hour exposure and 4508 cells in the 120-hour series. In the roots exposed for 140 hours the number of cells in mitosis is 125 in 4263 cells counted. The average percentage for the 140-hour period is 2.9, which is slightly lower than that observed for the untreated roots.

It appears from these studies that colchicine in a given concentration for a given period up to and including 24 hours increases the number of metaphase stages in the root-tips of the onion. The variations in individual responses for a given solution and a given period are approximately from 1 per cent to 3 per cent of the average for all the series.

It is clear that in these experiments .01 per cent colchicine has an inhibiting effect on the development of the prophase stage after the 24th hour. The extreme variation as shown in the graph indicates the beginning of the toxic effects exerted by the colchicine. This may be expressed in the increased toxic influence of the colchicine or the inability of the colchicine to keep the dividing cells in metaphase any longer. Both conditions seem to prevail. The colchicinized cells begin to degenerate; comparatively large lobulate nuclei are formed. In a subsequent report effects of a study of 900–3000 roentgen ray units on colchicinized root tips will be given.

SUMMARY

1. Onion root-tips grown in water for 10–15 days contain an average of 3.6 per cent cells in metaphase stage.

2. Roots exposed to .01 per cent colchicine from 6 to 24 hours contain a gradually increasing percentage of cells in metaphase which reaches the maximum at 24 hours.

3. Continued exposure for longer than 24 hours shows a gradual decline in the number of mitotic stages to a point slightly below the figure observed for the untreated root-tips.

4. The differences between extremes in the number of metaphase stages in untreated and in all colchicinized root-tips are approximately the same except at the critical exposures, shown here to be 24 hours with .01 per cent colchicine, where the variation is the greatest.

5. The number of metaphases in the colchicinized root-tips is predictable within the limits of variation of the 24-hour treatment as given here.

6. It appears that simple plant structures like the onion root-tip are suitable for the initial studies of the combined effects of colchicine and x-rays.

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DIFFERENTIATION IN RED ROOT-TIPS OF PHALARIS ARUNDINACEA

ROBERT BLOCH

Molisch[1] described the interesting occurrence of red root-tips in a number of plant families (Crassulaceae, Saxifragaceae, Balsaminaceae, Melastomaceae, Compositae); it was stated that the coloration was due to the presence of anthocyanin in the youngest part of the meristems and in the root-cap cells, but no further histological details were given.

During studies of developing roots of various genera of small-seeded grasses, the author noted frequent occurrence of a red coloration in root-tips of *Phalaris arundinacea*. The seeds were grown on damp lens paper in moist chambers with the new technique[2] for observation of living plant meristems at successive intervals. In the present study seeds were germinated either in light or in darkness, and red root-tips made their appearance after approximately four days.

Microscopic investigation of the living root showed that the entire meristematic area as well as the cells of the root-cap were intensely colored. The distribution of the pigment was always associated with a dense protoplasmic condition in the cells, but as the cells begin to vacuolate, at a distance of about 250–400 micra from the apex, the coloration becomes less conspicuous. At the last cell division in the basal portion of the meristem, two types of cells are set apart in the surface of the root; they are trichoblasts which later develop root-hairs, and ordinary surface cells which remain hairless. In *Phalaris arundinacea* these two cell types roughly alternate, though occasionally several hairless cells may occur in direct succession. Trichoblasts and hairless cells can first be distinguished by slight differences in the rate of longitudinal expansion (lower in the trichoblast), but in this genus the dissimilarity is made much more conspicuous by a difference in the depth of color, the trichoblast not only remaining more densely protoplasmic, but also more intensely colored; this condition often persists until the root-hair cell has reached considerable length (fig. 1). Even later some pigmentation was frequently noted still associated with the aggregation of cytoplasm at the base or the tip of the growing root-hair, which develops either at the apical end of the cell or somewhat more toward its middle. The much more rapidly expanding and finally considerably longer hairless cells, on the other

[1] **Molisch, H.** Rote Wurzelspitzen. Ber. Deuts. Bot. Ges. **46**: 311–317. 1928.

[2] **Sinnott, E. W.** Growth and differentiation in living plant meristems. Proc. Nat. Acad. **25**: 55–58. 1939. **Sinnott, E. W. & Bloch, R.** Changes in intercellular relationships during the growth and differentiation of living plant tissues. Am. Jour. Bot. **26**: 625–634. 1939.

hand, lose their dense cytoplasmic condition and their pigmentation at a much earlier stage (fig. 1).

The differences in the degree of pigmentation are obviously an expression of physiological, metabolic differences between trichoblasts and hairless cells in the transition zone located between the terminal meristem and the vacuolate, mature region of the root. The natural red pigmentation may serve as a tool in distinguishing without difficulty at a very early stage of differentiation the two types of cells in a living, undisturbed condition; it is equally of assistance in following their development in the region of vacuolation and maturation, and it makes it especially easy to identify the same cells when repeated observations of the same root tips are made.

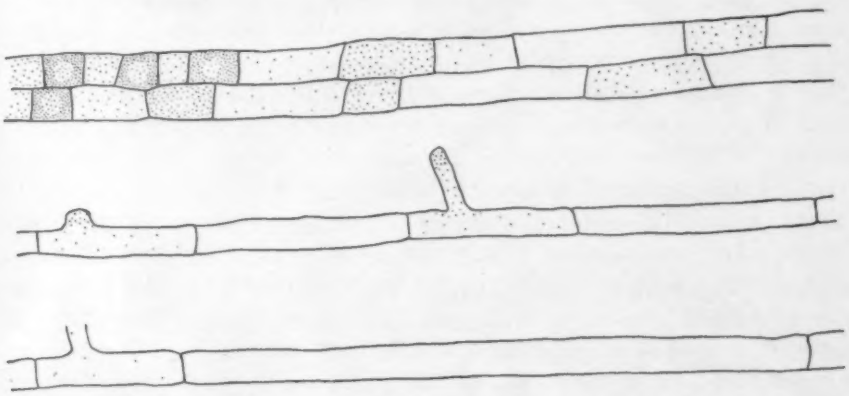

FIG. 1. *Phalaris arundinacea*. Surface of living root, showing unequal growth and the progressive loss of coloration in naturally pigmented trichoblasts (darker) and alternating hairless cells (lighter), in zones of beginning vacuolation (above) and maturity (below). × 250.

Generally the central portion of the densely protoplasmic cells appears lighter colored, indicating the position of the colorless nucleus. The color-giving pigment is apparently distributed in small vacuoles throughout dense aggregates of cytoplasm and becomes less conspicuous as general vacuolation proceeds in the cells; the color fades out first in the rapidly expanding hairless cells. The metabolically much more active trichoblasts retain much longer a considerable amount of cytoplasm and pigment, though these also progressively diminish. It follows that a trichoblast compared with an even younger hairless cell which has the same size may show a denser cytoplasmic content and a deeper pigmentation.

It was noted that in injured roots the coloration in the cytoplasm becomes at first more intense as a result of necrobiotic changes. As degeneration proceeds, however, the color is frequently taken up by the nucleus, which then turns darkish red.

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FURTHER EXPERIMENTS ON THE NUTRITION OF ISOLATED TOMATO ROOTS[1]

JAMES BONNER

INTRODUCTION

The exact accessory growth substance requirements of isolated tomato roots are at present in dispute. It is agreed by all the workers in the field that thiamine or a constituent portion or portions thereof are required. Robbins and Schmidt (1939 a, b) and Robbins (1941) have shown further that pyridoxine exerts a powerful growth-promoting influence on the growth of the isolated tomato root, a conclusion confirmed by Bonner and Devirian (1939), Bonner (1940), and Day (1941). White (1940) finds however that isolated tomato roots are not affected in their growth by pyridoxine but do respond to glycine (1939, 1940). Bonner and Devirian (1939) and Bonner (1940) could not establish any important effect of glycine but did find that in the presence of adequate amounts of thiamine and pyridoxine isolated tomato roots responded with increased growth to the addition of nicotinic acid. Neither Robbins and Schmidt (1939b) nor White (1940) confirmed this activity of nicotinic acid. Robbins (1941) has however later shown that different strains of isolated tomato roots vary greatly in their response to nicotinic acid, some responding vigorously and some responding little. The present work confirms Robbins and shows that closely related clones vary in their responses to nicotinic acid. In addition the responses of four clones of tomato roots to glycine are here reported.

METHODS

Each of the clones of isolated tomato roots discussed below was derived from an individual seed of *Lycopersicon esculentum*, var. "San Jose Canner." The seeds were disinfected in 0.1 per cent $HgCl_2$, and laid out to germinate in aseptic Petri dishes. When the seedling roots had attained a length of 2–3 cm. the terminal 10 mm. of root was removed and cultivated in 10 cc. Petri dishes containing 25 cc. of nutrient solution. The nutrient solution contained per liter of Pyrex-redistilled water: 236 mgs. $Ca(No_3)_2 \cdot 4H_2O$, 36 mgs. $MgSO_4 \cdot 7H_2O$, 81 mgs. KNO_3, 65 mgs. KCl, 20 mgs. KH_2PO_4, 1.5 mgs. ferric tartrate, and 20 gms. sucrose, with 0.1 mgs. thiamine, 0.5 mgs. nicotinic acid, and 0.1 mgs. pyridoxine as addenda. During the course of the experiments numerous trials were made in an effort to improve the consti-

[1] Report of work done with the assistance of the Work Projects Administration O. P. 365–1–07–2.

tution of the basic nutrient solution but no alteration of the amount of any chemical resulted in significant increase of root growth. Glycine where used was added at the rate of 3 mgs. per liter as recommended by White (1939, 1940). The roots were incubated in the dark at 25° C for one week and were then subcultured by removal of 1 cm. branch root-tips to fresh medium. This procedure of subculture and transfer was used in each subsequent transfer, which occurred at regular weekly intervals. At the end of each transfer period, the growth in length of the principal axis of each root was measured. The growth measurements presented below are averages of such measurements.

During the prosecution of this work, no piece of glassware which came in contact with a solution other than distilled water was used a second time without an intervening cleaning in sulfuric acid–potassium dichromate cleaning solution followed by seven or more rinsings in tap and distilled water, a precaution which has been observed in this laboratory since the initiation of isolated root culture work.

OBSERVATIONS

Uniformity of Material. Two hundred branch root-tips, randomly divided into 10 lots of 20 tips each, were used as the innocula for most of the experiments recorded below. Table 1 shows the degree of uniformity attained in a typical dummy experiment of this kind. Of the 20 initial tips, from 18 to 20 grew in the various groups. With respect to growth in length, the average deviation of the group means from the average of the group means was 3.8 per cent. Treatment of the data by the analysis of variance shows that the groups are homogeneous and do not differ significantly among themselves. The best estimate of the standard error of one group mean of 20 is 2.72 and correspondingly a minimum difference of about 20 per cent between two

TABLE 1

Uniformity trial with 200 one cm. branch root tips of isolated tomato roots (Clone W). Figures represent growth of principal root axis in mm. in 7 days

Group	Number of tips used	Number of tips which grew	Av. growth mm.	Std. error of av. growth
1	20	20	51.8	1.96
2	20	20	54.2	3.04
3	20	18	50.0	1.81
4	20	18	50.6	2.35
5	20	18	55.3	3.06
6	20	18	50.3	1.74
7	20	19	52.4	2.71
8	20	20	53.8	3.52
9	20	18	54.4	3.70
10	20	20	56.8	3.04

group means of 20 might be expected to be significant at the 1 per cent level. The work reported here is based on a series of 48 experiments generally similar in plan to that given in table 1.

Response to Various Growth Substances. In these experiments roots were cultivated for one week in nutrient solutions containing various addenda. The roots were then subcultured and cultivated for a second week in similar nutrient. Table 2 gives data on the growth, in this second transfer,

TABLE 2

Response of 3 different clones of isolated tomato roots to 6 different nutrient solutions

Added growth substances	Clone A	Clone H		Clone W	
	Expt. *G–44*	*G–33*	*G–34*	*G–31*	*G–41*
		Growth in mm. per root per week			
None	0.0	0.0	0.0	0.0	0.0
B_1 alone	0.0	0.0	0.0	0.0	0.0
$B_1 + B_6$	31.2 ± 1.58	31.3 ± 2.83	30.9 ± 2.37	46.4 ± 2.79	49.8 ± 2.55
B_1 + nicotinic acid + B_6	34.1 ± 1.60	40.3 ± 1.90[a]	38.8 ± 2.34[b]	44.3 ± 2.94	46.8 ± 1.91
Glycine alone	0.0	0.0	0.0	0.0	0.0
	G–43	*G–36*	*G–40*	*G–39*	*G–42*
None	0.0	0.0	0.0	0.0	0.0
B_1 alone	0.0	0.0	0.0	0.0	0.0
$B_1 + B_6$	48.4 ± 1.92	30.9 ± 1.28	28.2 ± 1.94	50.2 ± 2.22	49.4 ± 2.24
B_1 + nicotinic acid + B_6	50.0 ± 1.38	39.3 ± 2.06[a]	37.2 ± 1.35[a]	50.0 ± 2.28	46.0 ± 2.13
B_1 + glycine	0.0	2.5 ± 0.39	0.0	0.0	0.0

[a] Increase in growth rate associated with nicotinic acid significant at 1% level.
[b] Increase in growth rate associated with nicotinic acid significant at 5% level.

of three different clones in six different nutrient solutions. In no case was appreciable growth obtained in basic nutrient solution alone, in basic nutrient solution plus thiamine, or in basic nutrient solution plus glycine. In one experiment a slight response to thiamine plus glycine was obtained, but this could not be repeated in other experiments with the same clone. In every experiment thiamine plus pyridoxine supported good growth, whereas thiamine plus glycine elicited little or no growth. With these clones then, and with four additional clones of "San Jose Canner" roots for which detailed data will not be given, glycine does not appear capable of replacing pyridoxine, a conclusion in general agreement with that of Robbins. In other experiments, the influence of glycine in the presence of varied thiamine concentrations was investigated. In no case was response to glycine found.

Of the clones used in the experiments of table 2 only clone H responded significantly to the addition of nicotinic acid. This difference between the behavior of clone H and the other two clones (A and W) has been confirmed in 7 further experiments and it seems logical to conclude that individual

clones vary in their response to nicotinic acid. This conclusion is again in agreement with that of Robbins (1941).

One-Month Transfer Periods. The cultural conditions used by the present author differ in numerous ways from those used by Robbins and by White. Petri dishes rather than Erlenmeyer flasks are used as culture vessels and transfer periods of one week, following White, are used rather than the 1–2-month transfer periods used by Robbins. In one series of experiments therefore, tomato roots were grown in 125 cc. Erlenmeyer flasks, 50 cc. of nutrient per flask. The flasks were inoculated with basal fragments of vigorous roots and incubated in diffuse light for one month. These roots were then subcultured and incubated for a second month in fresh medium, after which the procedure was repeated for a third time. After each transfer dry-weight measurements (as used by Robbins) were made as well as linear growth measurements. Twenty roots of clone H (table 2) were measured and weighed in each treatment given in table 3, which presents data from the third

TABLE 3

Growth of roots of clone H in Erlenmeyer flasks during a transfer period of 1 month. Each mean is based on twenty roots

Growth substances in nutrient:	None	Thiamine	Thiamine + pyridoxine	Thiamine + pyridoxine + nicotinic acid
Av. growth mm. per root	0.0	5.2 ± 2.45	22 ± 5.75	58 ± 2.34
Av. dry wt. per root; mgs.	0.0	0.18 ± .09	2.0 ± 0.48	7.3 ± 0.28

monthly transfer. It is apparent that with this clone growth responses both to pyridoxine and to nicotinic acid were obtained under these conditions and that these responses were qualitatively similar to those obtained in Petri dish culture with one-week transfer periods. The responses are evident from the dry-weight measurements as well as from the linear measurements.

Small but definite growth in solutions containing thiamine as the sole added growth substance was observed in all three passages of this experiment. It appears quite possible that the same slow growth takes place in thiamine cultures of the present clones when these clones are transferred weekly but that owing to the small increments such growth can be detected only with difficulty.

Clone of P. R. White. Through the courtesy of Dr. P. R. White, samples of the standard White clone of isolated tomato roots were obtained on two occasions. In a typical experiment with this clone, of 44 roots maintained in thiamine–glycine medium all ceased growth within 3 transfers. Of 14 roots maintained in thiamine–nicotinic acid–pyridoxine medium all grew satis-

factorily and made an average increment of 32 mm. per root per week. It would appear that the White clone under the conditions used by the present author cannot be maintained in a thiamine–glycine nutrient medium.

DISCUSSION

The present work was undertaken in an effort to find answers to two questions: (1.) The reason for the discrepancy in the results of Robbins and those of Bonner as to the effectiveness of nicotinic acid as a growth substance for isolated tomato roots; and (2.) the reason for the discrepancy between the results of Robbins and Bonner on the one hand and those of White on the other as to the effectiveness of pyridoxine and glycine as growth substances for isolated tomato roots. As to nicotinic acid, it has now been shown both by Robbins and in the present paper that response to nicotinic acid (in the presence of thiamine and pyridoxine) is a property which varies with different clones or strains of isolated tomato roots. Some strains respond while others do not. Although the cultural conditions and the criteria of growth used in the two laboratories are different, still response to nicotinic acid can be determined, with suitable strains, in both laboratories. With regard to glycine, however, it does not seem probable that the different results obtained are due to metabolic differences in the clones used. Of 14 clones of isolated tomato roots which have been investigated by the present author, not one has been found in which glycine is able to replace pyrixodine, or in which glycine has yet been found to have any important growth effect. It would seem more probable that the effect of glycine is obtained under conditions peculiar to some laboratories and not found in other laboratories. Thus White (1940) found that Robbins' standard clone, when grown in White's laboratory, failed to respond to pyridoxine, but did respond to glycine although to a lesser extent than the White standard clone. White's standard clone as grown in this laboratory failed to respond to glycine in the presence of thiamine but did make satisfactory growth in thiamine–pyridoxine–nicotinic acid nutrient medium. It should be recalled also that White (1939) has found that sunflower roots make continued luxuriant growth in thiamine–glycine medium. In this laboratory sunflower roots were found to respond primarily to thiamine and pyridoxine (1940).

SUMMARY

1. Of three clones of isolated tomato roots reported in the present paper, all were found to grow luxuriantly through repeated transfers in nutrient solution containing thiamine and pyridoxine as accessory growth substances. None of the strains made continued growth in nutrient solution containing thiamine and glycine as accessory growth substances.

2. Of the three clones, one responded with increased growth when nico-

tinic acid was added to the medium in addition to thiamine and pyridoxine. The other two clones did not respond to nicotinic acid.

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THE RELATION OF CERTAIN FUNGI TO THIAMINE

WILLIAM J. ROBBINS AND ROBERTA MA

The discovery that certain fungi, incapable of growing on a basal medium, developed when traces of thiamine were added was followed by the observation that the growth-substance deficiency of some of them could be satisfied by substituting for thiamine its pyrimidine and thiazole intermediates.[1] This observation was interpreted in two ways.

On the one hand, it was assumed that thiamine was essential for all fungi. Some were autotrophic as far as this vitamin was concerned and synthesized it from elementary foods and minerals. Others were incapable of this synthesis and suffered from a thiamine deficiency which necessitated a supply of this vitamin in the nutrient medium. Those belonging to the group which grew in the presence of one or both intermediates were considered capable of synthesizing the essential thiamine from its intermediates and some of them were believed capable of making one of the intermediates as well. Robbins and Kavanagh (16) and Robbins (15) presented this point of view.

Other investigators, however, were inclined to believe that thiamine as such was not effective but owed its activity to some indirect action or to its intermediates. Schopfer and Jung (11) concluded that the action of thiamine on *Phycomyces* was indirect; its presence was considered indispensable for the synthesis by the fungus of factors peculiar to it. Schopfer and Jung (12) inclined toward the idea that *Phycomyces* split the thiamine molecule into its intermediates and used these for different purposes. Müller and Schopfer (3) believed that *M. ramannianus* probably split the thiamine molecule and utilized the thiazole portion. Schopfer (6) in studying the thiamine deficiencies of *Rodotorula* sp. concluded that these organisms split the thiamine molecule, thus liberating the thiazole or the pyrimidine which they required. Schopfer (7) admitted as a hypothesis that thiamine might be synthesized by fungi from its constituents but considered that the situation may vary with the organism.

The discovery that various species of *Phytophthora* needed molecular thiamine and failed to grow when furnished the intermediates, made possible a bioassay for thiamine (13, 14). By employing *Phytophthora cinnamomi* as a test organism Bonner and Buehman (2) demonstrated the synthesis by *Phycomyces* of thiamine from its intermediates. By using the same method of assay Schopfer (8) confirmed this observation and showed that

[1] The terms pyrimidine and thiazole as used here refer to 2-methyl-5-bromo-methyl-6-aminopyrimidine and 4-methyl-5-β-hydroxyethyl thiazole respectively. These are the two intermediates of thiamine.

Rhodotorula rubra grown on pyrimidine alone and *Mucor ramannianus* grown on thiazole alone also synthesized thiamine. Schopfer and Blumer (9) showed that *Schizophyllum commune*, which requires pyrimidine only as a supplement, formed thiamine in such a medium. From these reports, and those of others, it appears probable that the first of the interpretations presented above on the relation of fungi to thiamine is the more nearly correct.

In surveying the relation of species of *Ceratostomella* and other fungi to vitamins, several were found to suffer from thiamine deficiencies (18, 19, 20). It appeared worth while to determine for the members of this group the kind of thiamine deficiency concerned, and in view of the earlier differences in opinion to learn also whether thiamine or a physiologically equivalent substance was produced by those capable of growing on media supplemented by one or both intermediates.

METHODS AND MATERIALS

The fungi investigated were *Ceratostomella fimbriata*, *C. ips* No. 255, *C. microspora* No. C 109, *C. montium* No. C 424, *C. obscura* No. C 104, *C. penicillata* No. C 110, *C. piceaperda* No. 240, *C. pini* No. 416, *C. pini* No. 512, *C. radicicola* No. B 261, *C. stenoceras* No. 107, *Ceratostomella* from the London plane tree (*CLP*), *Chalaropsis thielavioides* No. C 117, *Endoconidiophora* (*Ceratostomella*) *paradoxa* No. 116, *Mucor ramannianus*, and *Polyporus versicolor* No. 71700 R.

Each organism was grown in triplicate in test tubes at 20° C on a basal solution solidified with 1.5 per cent purified agar[2] and supplemented with 5 mμ moles of pyridoxine and 0.05 μg. of biotin methyl ester, and on the same medium plus per tube 10 mμ moles of thiamine, of pyrimidine and thiazole, of pyrimidine, or of thiazole. Each test tube contained 8 ml. of medium.

All media were sterilized by autoclaving at 13 lbs pressure for 20 minutes. The thiamine and pyridoxine were Merck's synthetic and the pyrimidine and thiazole[3] were supplied by the same company. The asparagine was purified by treatment with Norit A and recrystallization from alcohol. The biotin methyl ester was obtained from the SMA corporation.

OBSERVATIONS

Types of Response to Thiamine and its Intermediates. Four of the fifteen fungi tested proved to be unable to use the intermediates but required

[2] The basal solution contained per liter 50 g. dextrose, 1.5 g. KH_2PO_4, 0.5 g. $MgSO_4 \cdot 7H_2O$, and 2 g. asparagine. To this solution the following trace elements were added in p.p.m. 0.005 B, 0.02 Cu, 0.1 Fe, 0.01 Ga, 0.01 Mn, 0.01 Mo, and 0.09 Zn. The agar was purified by extraction with 5 per cent pyridine followed by 0.1 N HCl and neutralization with $Ca(OH)_2$.

[3] Some samples of thiazole are contaminated with pyrimidine. We have been furnished such samples and have had reports to this effect from three other investigators.

thiamine as such. These were *Ceratostomella fimbriata, CLP, C. penicillata,* and *Chalaropsis thielavioides.* The response of *CLP,* typical of this group, is shown in figure 1 A after 15 days growth on the basal agar medium and on that supplemented with thiamine, the two intermediates, pyrimidine, or thiazole.

Five of the fifteen fungi grew satisfactorily on the basal medium supplemented with thiamine, or with the two intermediates, but failed to grow when the supplement was limited to pyrimidine or to thiazole. These fungi were *Ceratostomella ips, C. pini* No. 416 and No. 512, *C. radicicola,* and

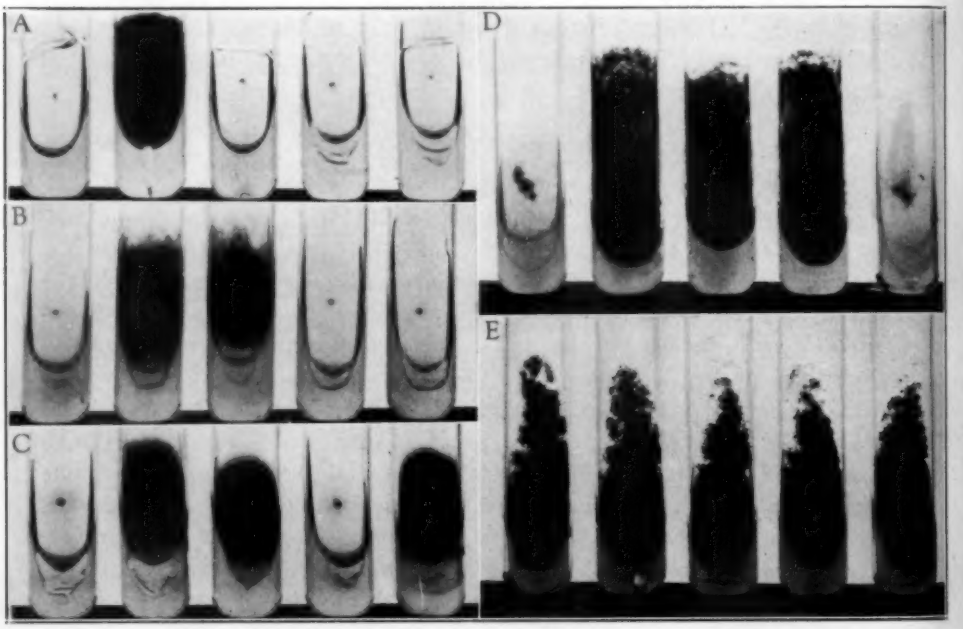

FIG. 1. Types of thiamine deficiency. Each fungus grown on a basal medium supplemented as follows: from left to right, nothing, thiamine, thiazole and pyrimidine, pyrimidine, thiazole. A, *Ceratostomella* from London plane tree; B, *Ceratostomella pini;* C, *Mucor ramannianus;* D, *Ceratostomella montium;* E, *Aspergillus niger.*

Polyporus versicolor. As far as could be judged from the growth on the agar slants, the two intermediates were as satisfactory as thiamine for these fungi. The growth of a typical representative of this group, *C. pini* No. 512, six days old, is shown in figure 1 B.

Six of the fungi grew on media supplemented with thiamine, with the two intermediates, or with pyrimidine, but did not grow on the basal medium or on the medium supplemented with thiazole. These fungi were *C. microspora, C. montium, C. obscura, C. piceaperda, C. stenoceras,* and *Endoconidiophora paradoxa* (fig. 1 D).

None of the fifteen showed a response like that of *M. ramannianus* (fig. 1 C) which grows on media supplemented with thiazole, but not on those supplemented with pyrimidine.

Quantitative Relations. The effect of various amounts of thiamine on the growth of *CLP* and *Endoconidiophora paradoxa* was studied briefly. These two fungi were grown in triplicate in test tubes containing 8 ml. of the basal agar medium supplemented per tube with 5 mμ moles of pyridoxine, 0.05 μg. of biotin, and 2 mg. of casein hydrolysate plus thiamine as follows: none, 0.001, 0.01, 0.025, 0.05, 0.075, 0.1, 0.25, 0.5, 0.75, or 1.0 mμ mole. At the end of eleven days the growth of *CLP* had covered the slopes in those tubes containing 0.25 mμ mole or more of thiamine. With smaller amounts growth was decreased (fig. 2). The effect of 0.001 mμ mole of thiamine was slight but visible. Under our experimental conditions 0.001 mμ mole of thiamine was slightly but detectably beneficial to *CLP* and quantities of 0.5 mμ mole or greater appeared to give the maximum benefit.

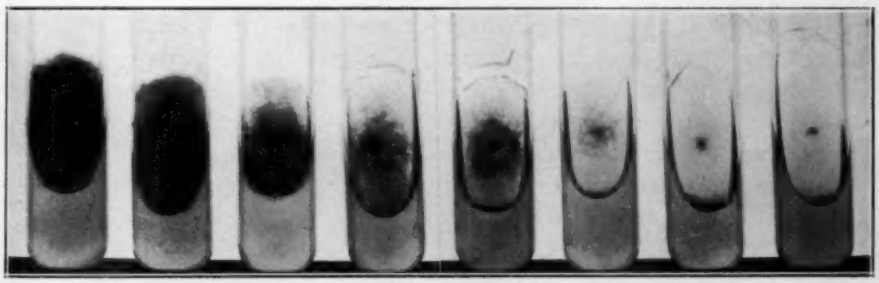

FIG. 2. Quantity of thiamine and *CLP*. From left to right, basal medium plus mμ moles of thiamine as follows: 0.5, 0.25, 0.1, 0.05, 0.025, 0.01, 0.001, none. Age, 11 days.

For *E. paradoxa* an effect of 0.001 mμ mole of thiamine was not observed, but 0.01 mμ mole was slightly beneficial. The growth increased in extent and heaviness up to 0.075 mμ mole of thiamine per tube.

While the growth of *CLP* or *E. paradoxa* on slants of an agar medium properly prepared could be used for approximating the quantity of thiamine in a substance to be assayed, the method is time-consuming and approximate only. It is doubtful whether further refinement would be desirable.

The daily growth rate in mm. of *Phycomyces blakesleeanus*, two strains of *E. paradoxa* (No. C 116 and No. B 520), and *C. radicicola* was determined in tubes in the presence of various amounts of thiamine after the method described by Beadle and Tatum (1). Fifteen ml. of the basal agar medium supplemented with pyridoxine, biotin and casein hydrolysate and quantities of thiamine ranging from 0.001 to 0.5 mμ mole were used per tube. In our experiments the growth rate of none of the fungi mentioned was sufficiently

correlated with the quantity of thiamine to suggest that it could be successfully used for determining thiamine by this method.

Synthesis of Thiamine from the Intermediates. The synthesis of thiamine by the fungi which grew on media containing the two intermediates was determined as follows:

Each fungus was grown in duplicate in 125 ml. Erlenmeyer flask containing 25 ml. of the basal solution supplemented per flask with 10 mμ moles of pyridoxine, 0.05 μg. of biotin, and 10 mμ moles each of pyrimidine and thiazole. Some flasks containing the same solution were retained uninoculated. After a moderate amount of growth had developed in the inoculated flasks (after from 5 to 14 days), acid produced by the fungus was neutralized with dilute KOH, sufficient purified agar was added to make a 1.5 per cent solution, and the flasks were autoclaved. At the same time agar was added to the uninoculated solutions which also were autoclaved. Both the check medium and that in which the fungus under investigation had grown were then inoculated with *CLP*. Since this organism does not grow unless the culture medium contains molecular thiamine, its development demonstrated the presence of thiamine or a physiologically equivalent substance. *C. ips*, the two strains of *C. pini*, *C. radicicola*, and *Polyporus versicolor* were found to produce appreciable quantities of thiamine when grown in liquid media supplemented with thiazole and pyrimidine.

In this method both the mycelium and the liquid in which the fungus under investigation grew were included in the final test medium. It is not possible to say whether the thiamine which was produced was retained by the fungus mycelium or excreted in part into the surrounding liquid.

Synthesis of Thiazole and Thiamine. The synthesis of thiazole[4] by those fungi which grew in a solution supplemented with pyrimidine was demonstrated by a procedure similar to that used in testing for thiamine production. However, the fungus investigated was grown in the basal solution supplemented with pyridoxine, biotin, and pyrimidine. *M. ramannianus* was used as a test organism for thiazole. The synthesis of thiamine also was determined for this group by using *CLP*.

Each of the six fungi which grew in solutions supplemented with pyrimidine only were found to produce thiazole and thiamine in a liquid medium supplemented with pyrimidine (fig. 3).

Synthesis of Pyrimidine and Thiamine. The synthesis of pyrimidine and thiamine by *M. ramannianus* was demonstrated by cultivating the fungus in the basal solution supplemented with thiazole. *E. paradoxa* was the

[4] The bioassays used in this investigation do not prove the presence of free thiazole or free pyrimidine.

test organism employed for pyrimidine and *CLP* for thiamine. Appreciable quantities of both pyrimidine and thiamine were found to be produced by *M. ramannianus* when grown in the basal solution supplemented with thiazole.

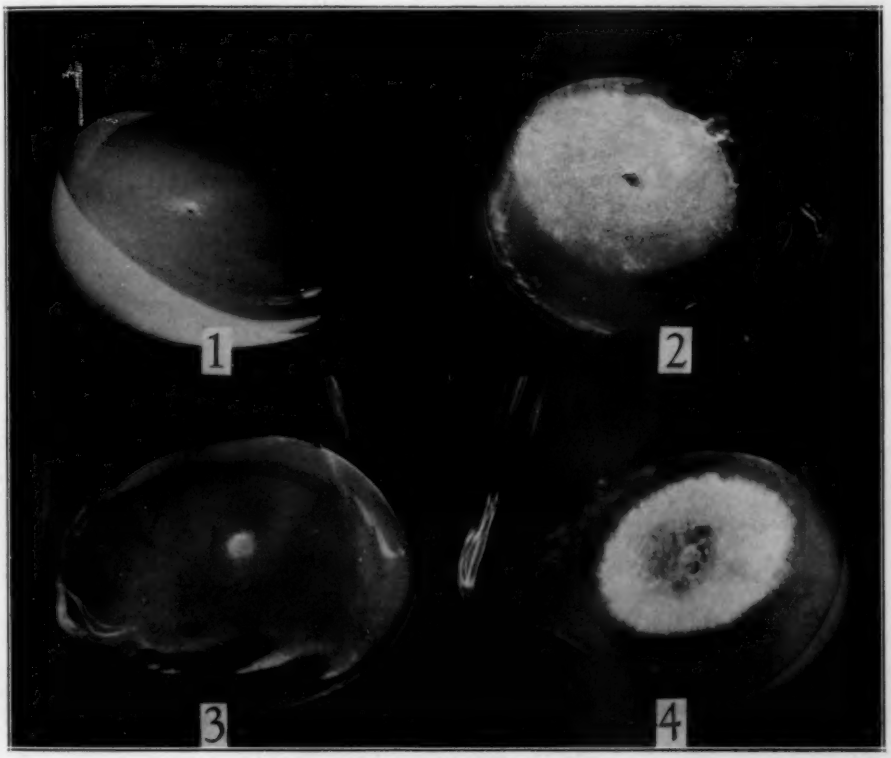

FIG. 3. Synthesis of thiazole and of thiamine by *Ceratostomella stenoceras* in a basal solution containing pyrimidine. (1) The basal solution supplemented with pyrimidine solidified with purified agar and inoculated with *Mucor ramannianus;* (2) basal solution supplemented with pyrimidine in which *C. stenoceras* was grown 11 days. After addition of agar and autoclaving inoculated with *M. ramannianus;* (3) same as (1) but inoculated with *Ceratostomella* from London plane tree (*CLP*); (4) same as (2) but inoculated with *CLP*. Age of cultures, 11 days.

DISCUSSION

Two of the organisms included in this report were previously studied. Schopfer and Blumer (10) found *Polyporus* (*Polystictus*) *versicolor* responded to a mixture of pyrimidine and thiazole, but not to one alone. Schopfer (4, 5) and Müller and Schopfer (3) found thiazole permitted growth of *M. ramannianus*, but pyrimidine was ineffective, and Schopfer

(8) reported that thiamine was formed by this fungus in a medium supplemented with thiazole only.

Combining the results obtained in this study, and those summarized by Robbins and Kavanagh (17), 19 filamentous fungi are now known which require thiamine as such (the majority of these are species of *Phytophthora*); 15, able to use the two intermediates; 30, which grow when supplied with pyrimidine alone. *M. ramannianus* remains as the unique example of a fungus which grows in a medium supplemented with thiazole only. We cannot explain why among thiamine-deficient fungi the ability to synthesize thiazole is so much more common than ability to synthesize pyrimidine. If we consider that thiamine-deficient fungi evolved from forms autotrophic for thiamine by a progressive loss in synthetic power, the course of events would appear to have been as follows: loss of ability to make one of the thiamine intermediates followed by loss of power to combine the intermediates into thiamine; and in such a sequence loss of ability to make pyrimidine would appear to have been far more frequent than to make thiazole.

Our results together with those obtained by others support the assumption that the bio-synthesis of thiamine occurs through the formation of pyrimidine and thiazole, and the combination of these intermediates into thiamine. If these processes are enzymatic, as is likely, it would seem that separate enzyme systems are concerned in the formation of thiazole, of pyrimidine, and in the union of the two to form thiamine. Otherwise it would be difficult to understand how the loss of ability to synthesize one of the intermediates could occur without at the same time causing loss of ability to synthesize another.

Are there fungi able to synthesize one or both intermediates but unable to combine them into thiamine? The existence of such organisms would be possible if independent enzyme systems are concerned in the synthesis of the intermediates and of thiamine, as suggested above, though the evolutionary sequence discussed previously would militate against their occurrence. If such fungi exist, and this is not probable, they would be included in the group which requires the addition of molecular thiamine to the medium, and they could be identified by the proper bioassays under conditions in which the amount of thiazole and pyrimidine in the original cultures was known.

In any event, the results of these investigations emphasize the importance of thiamine as an essential metabolite for the fungi.

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INDEX TO AMERICAN BOTANICAL LITERATURE

The aim of this Index is to include all current botanical literature written by Americans, published in America, or based upon American material; the word America being used in the broadest sense.

Papers that relate exclusively to bacteriology, forestry, agriculture, horticulture, manufactured products of vegetable origin, or laboratory methods are not included. If users of the Index will call the attention of the editor to errors or omissions, their kindness will be appreciated.

The Index is reprinted monthly on cards, and furnished in this form to subscribers at the rate of three cents for each card. **The different subjects as classified below may now be ordered separately** (but no orders will be taken for less than one year's issue in any classification). Correspondence relating to the card issue should be addressed to the Treasurer of the Torrey Botanical Club.

The Index to American Botanical Literature is classified under 8 headings as follows: Plant Taxonomy and Floristics (exclusive of fungi); Morphology (including anatomy, and cytology in part); Plant Physiology; Mycology and Phytopathology; Genetics (including cytogenetics); Paleobotany; Ecology and Plant Geography; General Botany (including biography). It is realized that some articles do not fall readily in any of these classifications, and users of the Index interested in a particular topic are requested to examine also classifications which may include closely related topics.

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